THE EFFECTS OF NARCOTICS AND OTHER SUBSTANCES ON TISSUE OXIDATIONS AND ON BIOLOGICAL ACETYLATIONS

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A Thesis

by

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
ADP	Adenosinediphosphate
AMP	Adenosinemonophosphate (Adenosine-5-phosphate)
ATP	Adenosinetriphosphate
ATP-ase	Adenosinetriphosphatase
CoA	Coenzyme A
2,4-DNP	2,4-Dinitrophenol
DPN	Diphosphopyridinenucleotide
DPN-ase	Diphosphopyridinenucleosidase
NAA	Nicotinic Acid Amide
OAA	Oxalacetic Acid
PL	Pigeon Liver Extract
RBH	Rat Brain Homogenate

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HISTORICAL INTRODUCTION

BIOLOGICAL ACETYLATIONS

Early Indications of Acetylation Phenomena.

The fact that acetylation took place in the animal organism was recognized as early as 1879, when Jaffe (1), and Baumann and Preusse (2) gave bromobenzene to a dog and isolated p-bromophenylmercapturic acid from its blood. Cohn (3) found that when a m-nitrobenzaldehyde was fed to rabbits, m-acetylaminobenzoic acid appeared in the urine; the para form gave rise to p-acetylaminobenzoic acid. Knoop (4) showed that the feeding of aminophenylbutyric acid to the dog led to the excretion of the acetyl derivative.

Hensel (5) attempted to ascertain the effects on acetylation of some compounds which might give rise to acetyl groups in the organism. These compounds were fed simultaneously with the compound to be acetylated (p-aminobenzoic acid or p-aminobenzaldehyde). He found that acetic acid increased acetylation by 60-340%, pyruvic acid by about 30%, and acetoacetic acid by about 20% whilst acetaldehyde caused no increase.

Harrow et al (6) found that ethyl alcohol increased the acetylation of p-aminobenzoic acid by 207%, the ethyl ester of acetic acid by 176%, sodium acetate by 158%, pyruvate by 126%,

and lactate by 22%. It was also found that insulin almost doubled the urinary excretion of the acetylated form. The effects of the above compounds were ascribed by Harrow to their effects on the combustion of carbohydrates.

The work of Klein and Harris (7) with rabbit liver *in vitro* is in agreement with the findings of Hensel, and Harrow *et al*, as regards the marked increase in acetylation by acetate. They showed that the enhancing effect of acetate far surpasses that of pyruvate, lactate, acetoacetate, or ethyl alcohol. However, Doisy and Westerfield (8) and Martin and Rennebaum (9) reported findings to the contrary. They demonstrated that acetate had a direct inhibitory effect on acetylation, while acetoin and pyruvic acid were found to cause a great increase. The foregoing merely indicates the impossibility of obtaining a clear picture of the acetyl precursor by balance experiments with intact animals, since there is no way of making the exogenous supply of precursor the limiting factor. The precursors of acetylation are available in abundant supply from metabolic reactions, and the yield of acetylated compound is not likely to be influenced to a great extent by the dietary addition of likely precursors of acetyl groups.

Isotope Studies

The use of isotopically labelled test substances provided the first direct evidence concerning the sources of

acetyl groups for acetylation. Bernard (10) showed that when deutero-acetic acid was administered to humans and rabbits, along with sulfanilamide, the acetylsulfanilamide eliminated in the urine contained 9 and 12% respectively, of deutero-acetyl groups. Similar results were obtained by Bloch and Rittenberg (151). Ethanol was also shown to be an acetyl precursor by the use of deutero-ethanol (11). On the other hand, deutero-succinic acid had no effect on acetylation (58). By employing carbonyl- and carboxyl-labelled pyruvic acid Anker (59) was able to demonstrate that only carbon atoms 2 and 3 are utilized for acetylation. Bloch and Rittenberg (151), in the course of investigations relative to the synthesis of cholesterol in the body fed the following compounds labelled with deuterium: butyric acid, alanine, n-valeric acid, and myristic acid. By the simultaneous feeding of amino phenylbutyric acid it was shown that all the compounds fed provided acetyl groups for acetylation since the acetyl-aminophenyl butyric acid formed in each case contained deuterium in good amounts. It was also found that only those compounds capable of providing acetyl groups for acetylation were able to form cholesterol. Deuteropropionic acid, for instance neither acetylated aminophenylbutyric acid nor formed cholesterol.

Sprinson (152) demonstrated that glycine labelled in the methylene group gave rise in the intact rat to acetic acid labelled in both carbon atoms. A plausible explanation

is that one molecule of glycine is degraded to formic acid, which condenses with a second molecule of glycine to give α,β-labelled serine as demonstrated by Sakami (153). Pyruvic acid, derived from serine, would be the precursor of doubly labelled acetic acid (59). These experiments demonstrate the existence of a mechanism in the body for the synthesis of acetate involving mono-carbon substances.

No attempt has been made to review here the extensive literature on intermediary metabolism, the rapid accumulation of which is a direct result of the introduction of the isotope technique into biochemical research. Such information can readily be obtained from appropriate review articles (61). The foregoing merely illustrates the central position of acetate in cell metabolism, of which acetylation reactions are an integral part. Individual acetylation reactions will now be discussed in some detail.

Acetylation of Aromatic Amines

The first in vitro demonstration of the acetylation of sulfanilamide was made by Klein and Harris (40) who, in 1938, incubated rabbit liver slices in a Ringer-bicarbonate or Ringer-phosphate medium containing sulfanilamide. They found that increased acetylation was achieved by the addition of glucose, pyruvate, lactate, and acetoacetate (in order of decreasing efficiency). There was little anaerobic acetylation as compared with the aerobic controls, although when acetate was added under anaerobic conditions the acetylation almost

reached the level obtained in the aerobic experiments. The authors drew the conclusion that the process was limited by the rate of production of acetate by the tissues, and might be increased by acetate or those substances giving rise to acetate in the course of their metabolism.

Lipmann (41), working with homogenates and extracts of pigeon liver, found acetate to be the most active acetyl donor, while acetoacetate and pyruvate showed half the effect of acetate. The results suggested that the immediate reactant was either acetic acid itself or a C_2 compound which arises more readily from acetate than from pyruvate or acetoacetate. In these experiments five times as much sulfanilamide was acetylated under aerobic as compared with anaerobic conditions. However, when ATP was supplied, the anaerobic level of acetylation was raised to that of the aerobic. This suggested the prominent part played by ATP in the acetylation reaction, and lent confirmation to the well-known fact that aerobic metabolism generates much more high-energy phosphate than does anaerobic metabolism.

The enzyme system responsible for the acetylation of aromatic amines was found to be water soluble. A partially purified enzyme system was prepared by Lipmann and Kaplan (46) and the following components were found to be necessary for optimum activity: acetate, ATP, coenzyme A, and cysteine.

Lipmann et al (60) in 1950, reported the separation of

pigeon liver extract into acetyl-donor and various acetyl-acceptor fractions by means of acetone precipitation in the cold. At pH 8.2, 40% acetone precipitated the acetyl-donor fraction together with the acceptor systems for acetoacetate and citrate synthesis. The sulfanilamide acceptor enzyme remained in solution but could be precipitated by increasing the acetone concentration to 60% (55).

The separation of the acetyl-acceptor enzymes from other components in pigeon liver extract has provided a means for the study of other acetyl-donor systems. To give one example, acetylphosphate displays quite a difference in behaviour in bacterial systems as compared with animal systems (154). Although acetyl phosphate has been found to function in anabolic reactions in bacterial systems, Lipmann was unsuccessful in obtaining an increase in the acetylation of sulfanilamide by synthetic acetylphosphate (41). Yet, Lipmann and Tuttle (42), using hydroxylamine as an acyl interceptor, claimed to trap what they presumed to be acetylphosphate formed by pigeon liver extract in the presence of ATP and acetate. Furthermore, the formation of the acetohydroxamic acid was shown to be dependent on the presence of Coenzyme A. The possibility was tendered that the ATP-acetate reaction gives rise to an acetylphosphate different from the synthetic compound. However, subsequent research (43,44) disclosed that synthetic acetylphosphate would serve as an acetyl-donor for sulfanilamide acetylation by the pigeon liver acceptor system

provided that extracts of Cl. kluyveri (45) were added. The nature of the required bacterial component will be discussed in a later section.

The sulfanilamide acetylation reaction has been most useful to those engaged in attacking the problem of the mechanism of acetylation. A reliable and sensitive chemical method is at hand for the determination of sulfanilamide; added to which acetylsulfanilamide, once formed, is extremely stable. In striking contrast are the difficulties encountered in the investigation of choline acetylation, which will be discussed next.

Acetylation of Choline

Hunt and Taveau (12) demonstrated as early as 1906 that the acetylation of choline resulted in a product of greatly increased pharmacological activity. However, it is quite understandable that so much time should elapse before it became known that acetylcholine (Ach) was a normal constituent of the body. There was little more reason to expect its presence than of any other pharmacologically active compound which physiologists employed to irritate the animal body. The one probable clue was the known presence of choline. In fact, Donath (157) had, in 1904, proposed a theory that implicated choline as the cause of convulsions in patients suffering from epilepsy.

The demonstration by Dudley (13) in 1929, that Ach was a normal constituent of the body was contingent upon the discovery by Loewi and Navratil (155) that physostigmine (eserine) inhibits the breakdown of Ach by cholinesterase, an active enzyme found in tissues. The presence of the choline ester in brain tissue was first demonstrated reliably by Chang and Gaddum (14) in 1933.

Synthesis of Ach by brain tissue was first reported by Quastel, Tennenbaum and Wheatley (15) in 1936. Rat or guinea-pig brain cortex slices respiring in a buffered medium to which glucose and eserine (to inhibit cholinesterase) were added, contained four to five times as much of the choline ester as the unincubated control. The synthesis was markedly influenced by the presence of oxygen. Very little was formed under anaerobic conditions, and cyanide inhibited the acetylation even when sufficient oxygen was present. Pyruvate and lactate could replace glucose under aerobic conditions. It was concluded that the synthesis of Ach took place as a result of metabolic changes in the tissue, and that the oxidation of the substrate provided the necessary energy.

Nachmansohn and Machado (16), in 1943, found that homogenized brain under anaerobic conditions would synthesize Ach, provided ATP was supplied. Under these conditions it was necessary to add fluoride to prevent the rapid destruction of ATP through ATP-ase activity. With the addition of choline and eserine as well as ATP they obtained with rat brain the

formation of Ach, but the yield was small and variable. However, when cell-free extracts were prepared by centrifuging the homogenates and discarding the particulate matter, it was found that the rate of Ach formation by the extracts was much higher, ranging from 35 to 100 ug. of Ach per hour per gram fresh tissue (16). The work of Nachmansohn and Machado provided the first direct evidence that ATP is involved in acetylation reactions.

Feldberg and Mann (17, 18), independently of Nachmansohn and Machado, showed that the choline-acetylating enzyme system is completely soluble in aqueous medium. They prepared cell-free saline extracts of acetone-dried brain which were capable of a high rate of synthesis under optimal conditions. It was found that in the presence of air (18) the formation of Ach by the extract was only half that obtained under anaerobic conditions. The addition of glutathione or cysteine to the system overcame the inhibition caused by air (19,20) and oxidized glutathione or cysteine inhibited the synthesis of Ach under both anaerobic and aerobic conditions. Iodo-acetate, copper ions, and iodine also inhibited the synthesis (16). On the grounds of the foregoing evidence Nachmansohn (16) postulated that the enzymes concerned in the acetylation contained -- SH groups.

For full activity of the water-soluble enzyme system, which can be extracted from acetone-dried preparations of mammalian brain, Lipton and Barron (21) list the following five

requirements:- choline; a suitable source of the acetyl group, such as citrate, cis-aconitate, or acetoacetate; potassium ions; ATP; and a coenzyme found to be present in boiled aqueous extracts of brewer's yeast or animal tissues. The presence of either cysteine or reduced glutathione is desirable with homogenates and acetone-powder extracts, particularly after dialysis or in the presence of oxygen (18,19,20).

The manner in which citrate enhances formation of Ach has been strongly debated. It has an activating effect on minces (27), homogenates, (20,21,28), and extracts (21,29,30) but appears to inhibit synthesis in extracts of acetone-dried squid ganglia (24). It has been claimed by Lipton and Barron (21) that citrate is more active than acetate as a precursor for acetyl groups in Ach synthesis. Barron and Perskey (31) suggested the following mechanism with citrate as substrate:

I. citrate $\rightleftharpoons$ oxalacetate + "active" acetate

II. "active" acetate + choline $\longrightarrow$ acetylcholine

It had previously been shown that citrate activation requires magnesium ions (32), which is in agreement with the above scheme, since Reaction I requires Mg^{++}.

Others have adopted the view that citrate activation is due to its effect of inhibiting ATP-ase activity by removal of divalent ions (24,33). However, ATP-ase activity in acetone-dried brain extracts is very low (34), and citrate shows a strong activating effect even in the presence of fluoride, when the ATP-ase activity must be negligible.

Another argument against the hypothesis that citrate acts by binding divalent ions is the fact that oxalate, which in this respect is similar to citrate, has been shown to be without effect on Ach synthesis (30).

Acetate had been reported by a number of authors (16,22,23,24,36) to have little effect on Ach synthesis, although positive results were achieved with the squid ganglia (24).. Nachmansohn et al (37), described a preparation from rabbit brain (purified by ammonium sulfate fractionation) in which acetate is essential for high activity, and calcium ions are required. This was confirmed by Balfour and Hebb (38) who have achieved the separation of two enzyme systems from rabbit brain, one utilizing acetate (plus calcium ions), the other citrate (plus magnesium ions). When the two systems are present together, citrate and acetate have equal effect on Ach formation.

Glucose may accelerate or inhibit the formation of Ach, depending entirely upon the conditions under which it is present, Glucose stimulates synthesis by brain slices or brain pulp, aerobically (22,23), while in extracts of acetone-dried brain both glucose and fructose cause a definite inhibition (17,18), which is due to the loss of ATP by hexokinase activity (39). However, when conditions are present for active glycolysis, glucose and fructose lead to the formation of large amounts of Ach by brain extracts (39). D-Glucosamine, which can be phosphorylated at the expense of ATP,

also effectively inhibits Ach formation in non-glycolizing extracts (39). The inhibition due to fructose and D-glucosamine can be relieved by the addition of N-acetylglucosamine which itself is not phosphorylated but exercises a strong inhibition on the phosphorylation of fructose and D-glucosamine, and thus prevents the loss of ATP (39). Klas-Bertil Augustinsson, (156) in a recent article entitled "Synthesis of Acetylcholine" states "that D-glucosamine inhibits choline acetylase in non-glycolyzing cell-free extracts; the presence of N-acetyl-glucosamine counteracts this effect." This is obviously a misinterpretation of the facts. The inhibition of acetylation by D-glucosamine is due only to a loss of ATP by hexokinase activity.

Pyruvic acid exercises a strong inhibition on the acetylation of choline under anaerobic conditions (20,29,30,35). Other keto acids tested, such as α-ketoglutaric (20,35), phenylpyruvic (20), hydroxyphenyl pyruvic (20), and oxalacetic (30) had similar effects.

Cholinesterase

As stated previously, there is present in tissues an active cholinesterase which rapidly hydrolizes Ach to acetate and choline. The enzyme is usually associated with the particulate matter of tissues, practically none being present in acetone powder extracts (20). However, most workers add eserine to their medium as a routine measure. Cholinesterase is inhibited by eserine as well as by diisopropyl fluorophosphate

(DFP) (158), and tetraethyl pyrophosphate (TEPP) (159), but none of these affects choline acetylase activity (24).

Assay of Acetylcholine

Ach is determined by biological assay. The various assay methods have been investigated by Chang and Gaddum (160) who found the frog rectus abdominis muscle and the longitudinal dorsal muscle of the leech the most satisfactory. The isolated ventricle of the clam has been used by several workers (161, 162). None of these methods is satisfactory, as a wide margin of error (approximately ± 10%) is involved. Hestrin (163) has recently described a method based on the formation of a hydroxamic acid by the reaction between Ach and hydroxylamine at alkaline pH.

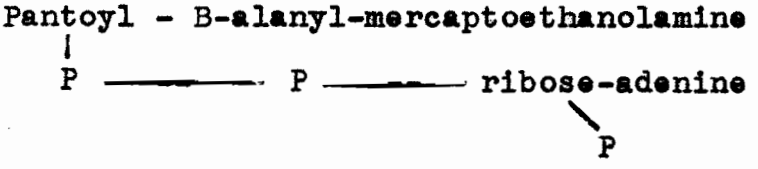
Coenzyme A

Feldberg and Mann (60,30) in 1945 reported a heat-stable dializable factor present in fresh brain extract, acetone-dried brain extract, and yeast, which, when added to a brain extract, greatly increased the synthesis of Ach. Nachmansohn and Berman (19) reported similar activity in a preparation which they obtained from boiled tissues (brain, liver, heart, and skeletal muscle). The active component was called "a coenzyme for choline acetylase". Lipmann and Kaplan (46,47) obtained by the fractionation of boiled pork liver, a factor which stimulated the acetylation of both sulfanilamide and choline. It was named Coenzyme A. The coenzyme is identical

with the "activator" of Feldberg and Mann (30), and that of Nachmansohn and Berman (19) and is a general constituent of living organisms.

Kaplan and Lipman (48) have described a method for the assay of Coenzyme A (CoA). The content of the latter in pigeon liver extract is destroyed by autolysis, thus making CoA the limiting factor in the acetylation of sulfanilamide. Reactivation of the extract is directly proportional to the CoA concentration. One unit of CoA is designated as that amount which reactivates their system to half the maximum activity.

Three major components are present in the coenzyme molecule: (a) pantothenic acid (49), (b) adenylic acid (49) and (c) an amino-containing sulfur compound, which is probably mercaptoethanolamine (49,50,51). The pantothenic acid is most likely linked to the sulfur-containing compound through the B-alanine carboxyl group (49,52), and to the adenylic acid by a phosphate bridge (53). There may also be a second phosphate grouping on the ribose of the adenylic acid. The following schematic formula is tentative:



There are strong indications that Coenzyme-A---enzyme complexes bind acetyl groups, as for example, by reaction

with acetyl phosphate (54). This possibility arises from the fact that purified enzyme fractions from Cl. kluyveri (45) contain a transacetylase which makes acetyl phosphate available as an acetyl-donor to Coenzyme A - dependent liver acetyl-acceptor systems (55). Lynen and Reichert (56) have produced evidence indicating that the function of Coenzyme A is actually that of a transacetylase. By fractionation of a boiled suspension of baker's yeast he obtained an impure preparation which contained Coenzyme A acetylated at the sulfur atom. This acetyl-CoA preparation when added to liver extract was capable of sulfanilamide acetylation.

Two-Carbon Fragment Generation

The first suggestions of acetate formation in animal metabolism go back to the experiments of Dakin (67) and Knoop (68) on the biological degradation of fatty acids. Thunberg (69), in 1920, postulated that the oxidative decarboxylation of pyruvate gave rise to acetate. He also pointed out the probability that the metabolic paths of fat, carbohydrate and protein converge at the two carbon stage, from which only one common mechanism of oxidation would be required.

Schoenheimer and Rittenberg (70) demonstrated the stepwise degradation of a biologically occurring fatty acid by elimination of two carbon units. Although the two-carbon units were not identified as such, their intermediary formation was established by Weinhouse, Medes, and Floyd (71), who labelled

octanoic acid at the carboxyl group with C^{13}, and showed that the acetoacetic acid formed from this compound in liver slices must have been formed, in part, by random condensation of C_2 fragments. It is fairly well agreed that the formation of a C_2 fragment is an obligatory step in the breakdown of fatty acids, and that acetoacetate formation in liver tissue is due to inability under certain conditions of that organ to dispose of the C_2 fragments by oxidation. This point is well exemplified in the experiments of Lehninger (72) who found that the addition of fumarate to preparations of washed liver cells oxidizing octanoate, diminished the amount of acetoacetic acid formed and increased the concentration of intermediates of the tricarboxylic acid cycle. On the other hand, Lehninger (73) described a malonate-insensitive system in liver which forms acetoacetate from pyruvate, aerobically. Lehninger's system is "malonate-insensitive" in the sense that blocking the tricarboxylic acid cycle forces an alternative disposal of the C_2 fragments arising from pyruvate, which in this case are directed towards acetoacetate formation.

The evidence to the effect that pyruvate is a source of C_2 groups is indisputable. Pyruvate enhances the formatinn of Ach (15), the acetylation of aromatic amines (6,7,8,9,41), the formation of acetoacetate under certain conditions (73), and the production of citrate (75). Acetate has been shown to accumulate as a result of pyruvate breakdown in animal tissues, both aerobically (75,76,77,78) and anaerobically by dismutation (76,77). The oxidation of pyruvate by dialized dispersions of

pigeon brain gives rise to the accumulation of citrate and α-ketoglutarate provided fumarate is present, while in the absence of fumarate the oxidation of pyruvate is associated with the formation of acetate (75). The oxidation of pyruvate by kidney or liver slices in the presence of fluoroacetate leads to the accumulation of acetate as a result of inhibiting the further oxidation through the tricarboxylic acid cycle (105). Lehninger's work (72,73) indicates that pyruvate supplies a precursor common to the formation of acetoacetate and citrate, which precludes the possibility that oxidative decarboxylation of pyruvate and condensation with oxalacetate are interdependent processes, but rather points to the obligatory formation of an active acetyl group indistinguishable from that which is active in acetylation reactions.

Mechanism of Acetylation

The discovery that Coenzyme A was a common factor in the acetylation of sulfanilamide, and the formation of acetoacetate and citrate, indicated that the same precursor was involved in all three reactions. Soodak and Lipmann (65) using the same preparation of liver extract as was used in the acetylation of sulfanilamide, observed a synthesis of acetoacetate from acetate and ATP. CoA was shown to be necessary. Furthermore, a common acetyl precursor was indicated by the fact that sulfanilamide inhibited the formation of acetoacetate. Stern and Ochoa (63) found that, in extracts of pigeon liver, citrate was readily formed from acetate and oxalacetate in the

presence of ATP, CoA and Mg^{++}. Also, since oxalacetate markedly depressed synthesis of acetoacetate and the acetylation of sulfanilamide, the same acetyl derivative was probably involved in the three condensations. Stern and Ochoa also showed that pyruvate is probably oxidized to acetate rather than participating *per se* in the condensation with oxalacetate, thus lending support to the implications of Lehninger's experiments (72,73). The catalytic function of CoA in citric acid synthesis was confirmed by Novelli and Lipmann (44) in experiments with cell-free extracts of yeast, and *E. coli*. Furthermore synthetic acetylphosphate was shown to act as acetyl precursor in citric acid synthesis by extracts of *E. coli*, due to the presence in these extracts of a CoA dependent transacetylase (46) which converts acetylphosphate into an active acetyl donor.

The failure of animal enzyme preparations to utilize synthetic acetylphosphate as an acetyl donor can be attributed to the absence of phosphotransacetylase (45). However, the addition of bacterial extracts, containing this enzyme, to animal preparations containing acetyl-acceptor systems will enable the latter to utilize acetyl phosphate (106,165).

From the data accumulating during the last few years (44,63,65,165) it appears in all likelihood that the so called "active acetate" is acetyl-CoA (54,56), which may be derived from a variety of enzymic reactions, and in turn may donate its acetyl residue to a variety of acceptor systems. Thus Coenzyme A acts as an acetyl-carrier, in much the same way as adenylic

compounds function as phosphate carriers.

The functional group of Coenzyme A is the -SH group. It has been suggested (56) that the acetylation reaction is initiated by a transfer of a phosphate group from ATP to the S atom of Coenzyme A, followed by the exchange of the phosphate group for acetate giving acetyl-CoA ($R-S-CO-CH_3$).

THE MECHANISM OF NARCOSIS

The scientific investigation of narcosis began in the year 1846, when Morton and Jackson introduced ether into surgical practice. The earliest names associated with this investigation are those of Bibra and Harless, in 1847, who observed that cerebral fats are soluble in ether and chloroform, and used this as a basis for a hypothesis of the mode of action of narcotics. The same relationship between the known narcotics of that day and their solubility in fats and oils was also observed by others but no definite correlation was clearly established until 1899 when Hans Meyer (109) proposed the "lipoid theory of narcosis". It was formulated in three phrases:

(1) Toute substance chimiquement indifferente et soluble dans les graisses et les lipoides, est un narcotic;

(2) L'action se manifestera en premier lieu dans les cellules dans lequelles les lipoides ont des fonction importantes, c'est-a-dire dans les cellules nerveuses;

(3) L'activite depend du coefficient de partage, qui determine la repartition de ces substances dans un melange d'eau et desubstances lipoidique.

Independently, the botanist Overton (110) as a result of his work on cellular permeability, came to the same conclusion.

The lipoid theory implies that the narcotic properties of compounds vary in the same fashion as their lipoid solubilities, and in the form put forward by K. H. Meyer (113), that equal depths of narcosis are produced by different substances when these are present at the same molecular concentration in the lipoids. Winterstein (112) has emphasized that the lipoid theory is really a theory of narcotics rather than of narcosis, while Kürt Meyer (111) during a symposium which dealt with narcosis, pointed out that, "It is not really a theory which explains the mechanism of narcosis, but rather the expression of an experimentally observed regularity, a rule of which every theory must take account." Verworn (118), in 1911, also pointed out that the lipoid theory was not a theory of narcosis since it did not explain the mechanism of narcotic action. However, the proponents of the theory held the belief that the lipoids of the cell are involved in the action of the narcotics.

Verworn (118) presented the first theory of narcosis to take into consideration the possibility that metabolism was

involved. He demonstrated that narcosis was accompanied by a drop in oxygen consumption of the cells, which he attributed to "asphyxia". Warburg's adsorption theory (119) also associated narcosis with oxygen lack. Warburg showed that the oxygen uptake of various cells was reduced, the extent depending on the narcotic concentration. This effect was explained on the grounds that differential adsorption of the narcotics displaced enzymes from vital surfaces. However, Warburg's charcoal model provided no evidence that there was any significant adsorption of narcotics on "vital surfaces" in the cells of the living organism.

The Cellular Membrane Theory of Narcosis

Höber (166), Lillie (167), and Winterstein (168), proposed theories which had in common the assumption that narcosis is due to a decrease in cell permeability. Lillie (120) as a result of experimental observations that narcotics either decreased permeability or prevented the increase of cellular permeability, expounded a popular theory based on the assumption that narcotics prevent the depolarization of the nerve membrane by abolishing the increase in permeability upon which depolarization depends. However, Lillie's theory was not substantiated by the investigations of other workers (121,122).

The effect of narcotics on polarized membranes has been shown most effectively in the case of peripheral nerve fibres. Blockage of the nerve impulse is believed to be the

result of stabilization of polarization (123,124,125) or by depolarization (125). How these effects are produced by narcotics has never been demonstrated, but attempts at explanation have always emphasized change in permeability of the membrane. A very ingenious experiment was devised by Larrabee et al (126) to compare the effects of narcotics on neuronal metabolism and synaptic transmission by simultaneously measuring the oxygen consumption and transmission in excised superior cervical sympathetic ganglia of rabbits. A ganglion was placed in a flowing solution in a small chamber in which oxygen concentration was measured with a polarized platinum electrode. The rate of oxygen consumption was determined by measuring the rate of fall of oxygen concentration when the flow of solution was stopped. The preganglionic nerve was stimulated and the transmission was measured by the height of the postganglionic action potential produced. All anaesthetics tested except urethane (chloretone, nembutal, ether, chloroform, and alcohols from methanol to octanol) depressed synaptic transmission without slowing the resting oxygen consumption. At sufficiently high narcotic concentration oxygen consumption was markedly inhibited. When the oxygen consumption was depressed by nembutal or chloretone, it could be restored by methylene blue, but depressed transmission was not affected. The extra oxygen consumption caused by repetitive activity was inhibited by the narcotic in direct proportion to depression of activity. The resting rate of oxygen consumption was inhibited markedly by azide and cyanide before transmission was affected. The extra oxygen consumption

of activity was depressed by cyanide more than the activity itself. The conclusion was drawn that narcotics depress neurones by some mechanism which does not interfere with oxygen consumption.

This conclusion is not valid on the basis of the evidence presented. There are indications that two oxidative processes are present in nerve tissue, one of which is related to excitation, and the other not. The resting oxygen consumption is presumably not related to excitation. The restoration of oxygen consumption which has been depressed by a narcotic can be restored by methylene blue, but no high-energy phosphate is built up in the process. The main criticism lies with the fact that the cohclusions are based on measurements of total oxygen consumption. When narcotics act upon the respiratory system of the cell they do so at special points in the chain (129). If a narcotic, at low concentration, is exercising a large inhibitory effect on an aspect of a respiratory chain that is linked with ATP synthesis, it does not necessarily follow that an equally large inhibitory effect on the total oxygen uptake will also be observed. The step inhibited by the narcotics may only represent, in terms of total oxygen absorbed by the system, a small fraction of the entire process. To illustrate, a low concentration of Nembutal which inhibited the oxygen consumption of brain slices by only 15%, caused a 50% decrease in the synthesis of acetylcholine, which in turn is a reflection of the inhibition of ATP formation (130).

Theories of Narcosis Based on Inhibition of Oxidation

Verworn (118) was the first to suggest that the phenomenon of narcosis is linked with inhibition of oxidation. His insight was remarkable when considered in the light of the paucity of biochemical knowledge and experimental techniques of his day. Some of his remarks made in 1911 when he delivered his Harvey Lecture on Narcosis are worthy of recapitulation:

"The factor which produces the characteristic symptom complex of narcosis is under all circumstances the suppression of the power to carry on oxidations ... When we recall the fate of molecular oxygen in the normal metabolism of the cells, from the moment at which it enters the living substance to the moment at which it decomposes the oxidizable materials into carbon dioxide and water, we find that the narcotic, which overflows the cell, could establish the inhibitory action upon the oxidation at various stages of this process ....

"However, I wish to emphasize again, that the conception regarding the nature of inhibition of oxygen metabolism in narcosis is of a purely hypothetical character. It is only an established fact that narcotics induce an acute asphyxia of the cells. Herein, is the essence of narcosis."

Verworn stressed the fact that narcotics inhibit oxidative processes; his use of the word asphyxia was purely figurative.

The modern concept of a mechanism of narcosis based on inhibition of oxidative processes was formulated by Quastel

and Wheatley (131) in 1932. The authors were able to show that narcotics inhibit the oxidation of glucose, lactate, and pyruvate by brain tissue, and that the oxidation of succinate was unaffected by the narcotic. The early work of Quastel and Wheatley was confirmed by Jowett and Quastel (132), and extended to show that inhibition of oxidation was not restricted to the brain, or to carbohydrates and their breakdown products. It was found that when rat or guinea-pig brain slices respired in the presence of a narcotic (pentobarbital, chloretone, or evipan) the inhibition of oxidation was greatest when glucose was being oxidized, followed by pyruvate and lactate; glutamate inhibition was smaller, while the oxidation of succinate was unaffected. These findings were a confirmation of the early results of Quastel and Wheatley, who used minced whole brain under somewhat different conditions. It should be mentioned that when n^{o}_{Λ} oxidizable substrate was added, the small oxidation of the brain slices was not appreciably affected by the narcotic; this residual respiration was shown to be due largely to substances other than glucose, lactate or pyruvate. That the narcotic was not competing with the substrate for the enzymes involved was indicated by the fact that substrate concentration did not influence the degree of inhibition by the narcotic. Of some importance was the finding that narcotic concentrations which produce narcosis in vivo are of the same order of magnitude as those which inhibit measurably the respiration of the cerebral cortex in vitro. In making the comparison it should be remembered that the in vitro inhibitions represent the effects of the narcotics on the

respiration of the entire brain cortex of the animal, while *in vivo* the inhibitions will be much higher at those parts of the nervous system where the narcotic is localized or specifically absorbed.

The effects of narcotics such as the barbiturates, chloretone, or hyoscine, on the respiration of rat brain slices are reversible (133). On the contrary, the effects of ether are irreversible (134) as are those of indole, which is a powerful inhibitor of brain respiration (133). Irreversibility may be explained, conceivably, on the basis of irreversible denaturation of the proteins with which these substances become associated.

It has been suggested that the mechanism of reversible denaturation might account for the observed effects of narcotics and other substances on metabolic processes and thus induce a state of narcosis (137). (If this is so, we are still confronted, with the question of the manner in which the reversible denaturation takes place). The theory is held by some that an equilibrium exists between the native and denatured forms of a protein-enzyme, the native form being the active one. The narcotic is believed to shift the equilibrium in favour of the denatured form. The basis of this theory is the effect of temperature and pressure in reversing the narcotic effect of some drugs on the luminescence of bacteria (135,136,137). Such a theory is compatible with the need to explain the action of narcotics in terms of physico-

chemical rather than chemical effects when one considers the wide variety of structural types of compounds which have narcotic action.

We have seen that the oxidation of succinate is not inhibited by concentrations of drugs that do inhibit the oxidation of glucose, lactate, and pyruvate (131). This indicates that cytochrome oxidase and succinic dehydrogenase are unaffected by narcotics, since both are required in the oxidation of succinate. By the use of various techniques it was possible, through the process of elimination, to determine which section of the respiratory chain was narcotic sensitive. Quastel and Michaelis (140) demonstrated the insensitivity of the dehydrogenases of brain, muscle, and yeast to chloretone by showing that anaerobic oxidation of substrates in the presence of ferricyanide and pyocyanine was unimpaired. Dixon and Zerfas (141) by the use of methylene blue, showed that the oxidation of DPN.2H by flavoprotein was not inhibited by narcotics. This had been previously demonstrated by Quastel and Michaelis (140) who used a system consisting of muscle extract, lactate, DPN, heart flavoprotein and methylene blue, and found that chloretone had no effect on this system. It was suggested by these authors that a flavoprotein which plays an intermediate role between DPN.2H and cytochrome oxidase is the narcotic-sensitive step. Greig (142) has confirmed the findings of Quastel, et al, and suggests that in the presence of the narcotic there is a binding of reduced flavoprotein with cytochrome b or other intermediates. There is sufficient evidence to

indicate that a special component of the respiratory system is singularly narcotic sensitive.

Respiration and Phosphorylation

Respiration is closely connected with the generation of high-energy phosphate (143). The inhibition of respiration may be expected to influence greatly the esterification of inorganic phosphate with a resultant drop in ATP formation. Eiler and McEwen (144) have shown that pentobarbital inhibits the generation of high-energy phosphate bonds to the extent that it interferes with oxygen utilization. With pyruvate as substrate, pentobarbital inhibits both respiration and phosphate uptake proportionately. It has been shown that narcotics do not interfere with anaerobic formation of acetylcholine by beef brain extracts and therefore have no effect on the phosphorylation involved in this process (145). The narcotics definitely suppress the aerobic formation of acetylcholine, presumably by the suppression of ATP formation. Brody and Bain (146) on the basis of their experimental findings postulated that an uncoupling of phosphorylation from oxidation may be one of the ways in which barbiturates act to produce anaesthesia, while Perskey, Goldstein, and Levine (147) found that pentobarbital blocks the pyruvic acid oxidase system, most likely at the two-carbon fragment. They stated that "the barbiturate does not block the oxidation of flavo-protein by either oxygen or cytochrome c. Rather, essential sulfhydril groups of the dehydrogenase of the pyruvic acid system are

shown to be the barbiturate-sensitive step."

Rosenberg et al (149) have shown that narcotics (chloral, chloretone, neonal, and phenobarbital) augmented by about 50% the aerobic consumption of glucose by rat brain slices, and at the same time the formation of lactic acid was increased to 180% or more. When respiration was diminished by 50%, aerobic glycolysis was augmented by 200 to 300%. This work lends confirmation to that of Hutchinson and Stotz (148) and of Greig (142). Independently of Rosenberg et al, Webb and Elliott (150) showed that the maximum aerobic glycolytic rate, equal to or exceeding the normal rate of anaerobic glycolysis, occurred when the oxygen uptake was about 50% inhibited by narcotics. In contrast to the above findings are those of Perskey, Goldstein, and Levine (147) who reported that pentobarbital completely inhibited both aerobic and anaerobic glycolysis at a concentration of 4mM/l.

Effect of Narcotics on Resting Level, and Activity Level of Oxidation

A number of investigators (180,181) have postulated that the total oxygen consumption of diverse types of cells results from two oxidative chains, which correspond to the "resting" level and the "activity" level, respectively, of oxygen consumption. Recent work (127,128) indicates that in nerve tissue there are two oxidative processes, one of which specifically increases as a result of excitation, and one of which does not. Herein probably lies the explanation for the

fact that in Larrabee's sympathetic ganglia preparation (126) the narcotic concentration which blocks transmission of the stimulus fails to depress the resting level of oxidation. Since only the activity level of oxidation is associated with excitation, it is quite logical that when the activity level of oxidation is depressed by a narcotic there will be no transmission. An analogous case is that of luminous bacteria, in which there are indications of two oxidative processes, one of which, the"activity system", is associated with light production.

Jowett and Quastel (132) some years ago, observed that the respiration of brain slices in the absence of added substrate was but little affected by a narcotic concentration which inhibited oxidation in the presence of added glucose by 57%, while doubling the narcotic concentration inhibited respiration markedly in the former case.

Recently, McIlwain (182,183) provided evidence that concentrations of atropine and hyoscine which had no effect on the "resting level" of oxidation inhibited the "activity level" of oxidation evoked by electrical stimulation of brain slices in vitro. Moreover, it was observed that stimulation of oxidation by other means (high KCl, 2,4-dinitrophenol) was not affected by concentrations of atropine and hyoscine much greater than that necessary to inhibit by 50% the increased oxidation due to electrical stimulation. The stimulation in vitro is cons idered to be similar to that occurring during

increased activity of the central nervous system *in vivo*.

Narcotics have in common the ability to depress reversibly particular functions or activities of individual cells as well as of whole organisms. This ability is well illustrated by their effects on nervous function, cell division in sea-urchin eggs, light production in luminous bacteria, sperm motility, photosynthesis, etc. Claude Bernard, in 1875, emphasized the fact that anaesthesia, which is a special example of the general phenomena of narcosis, is a universal phenomenon, and not peculiar to the nervous system alone, nor even to animal organisms. However, the distinguishing characteristic of a narcotic or anaesthetic must be its effect on nervous function. The necessity arises from the fact that in recent years many substances have been found which can depress function or activities of cells but which do not have narcotic activity, i.e. they do not induce hypnosis or anaesthesia in the animal organism. One such group of substances is the nitrophenols.

It is likely that the numerous effects produced by anaesthetic drugs on forms of life widely different from one another can be attributable to a common mechanism. It is extremely unlikely that these effects are unrelated to narcosis as defined in terms of reversible suppression of nervous excitability. Subsequent research, we might suggest, will vindicate Bernard's concept of a universal mechanism (114).

Henderson (107), in 1930, concluded an extensive review on narcosis with the words: "the final chapter in the theories of narcosis has yet to be written." Butler (108) in 1939), on the same subject, could still agree with Henderson, and went so far as to question the advisability of seeking a theory with universal validity. The root of Butler's scepticism is the apparently insuperable task of correlating the widely divergent effects of narcotics. The problem, however, is more apparent than real. A universal mechanism of narcosis does not demand that every substance which shows narcotic properties must have the same primary point of attack. If it can be shown, for instance, that the direct cause of the narcotic effect is a blockage in the production of energy required to sustain the particular activities affected by the narcotic, the requirements for a general mechanism will have been met. This type of evidence is accumulating in the field of biochemistry.

EXPERIMENTAL METHODS AND PROCEDURES

TISSUE PREPARATIONS

Beef Brain Acetone Powders

Beef brains were obtained from the abattoir within 30 minutes of the death of the animals.

The gray matter was removed, rinsed free of blood in ice-cold distilled water, and dried with filter paper. It was then suspended in its own volume of an ice-cold neutral solution of the following composition: Nicotinic acid amide (0.16M), cysteine hydrochloride (0.013M) and disodium hydrogen phosphate (0.02M). The suspension was homogenized for 30 seconds in the Waring blendor, after which it was poured into 10 volumes of ice-cold acetone. At the end of 5 minutes the dehydrated material was filtered, washed well with acetone, partially dried by suction, and stored as a dry powder in a vacuum desicator at 0°C.

Care must be taken during the washing process that air is not sucked through the powder when the acetone has been removed as this results in a brown powder, the activity of which is very poor. Experience has shown that the whiteness of the finished product bears some relationship to its activity.

The presence of nicotinamide (NAA) during the preparation of the powder protects the DPN to a great extent against destruction by DPN-ase in spite of the fact that the NAA is removed by the acetone. In any case, NAA was always added to the extraction fluid when the preservation of DPN was desired.

Extracts of the acetone-powder were made by rubbing up the powder in an aqueous solution of various salts as the purpose required. After centrifuging the extract for 15 minutes at 1800 r.p.m. in an angle centrifuge the supernatant was filtered through glass wool, and pipetted directly into the Warburg manometer flask.

Pigeon Liver Acetone Powder

The pigeons were decapitated, the livers immediately removed and rinsed free of blood with ice-cold distilled water.

The livers were first minced with stainless steel scissors followed by homogenization for 60 seconds in the Waring blendor. To facilitate the homogenizing, ice-cold acetone was added to the minced tissue in the Waring blendor in sufficient quantity to submerge the blades. (It has been observed that excessive treatment with the Waring blendor results in decreased activity of the powder).

After homogenizing the minced tissue the resultant fine suspension was added to 10 volumes of ice cold acetone. At the end of 5 minutes the dehydrated material was filtered on a Buchner funnel and washed well with acetone. Care was taken

not to allow air to be drawn through the material when the last of the acetone has disappeared. The dehydrated pigeon liver was removed from the Buchner funnel as a solid cake, which was placed on a large circle of filter paper and broken up into small pieces, with a spatula. Drying was completed in a vacuum desiccator over calcium chloride. The product was a light pink in color.

The powder retained full activity for well over a month when stored in a vacuum dessicator at 0°C. Some preparations retained activity for a period of 3 months.

Not all preparations displayed the same acetylating activity, even when prepared in the same manner. The pigeons, obtained from a local dealer, were not selected as to age, size, etc. which was no doubt conducive to a lack of uniformity in the preparations obtained. It was also observed that powders prepared during the winter months had somewhat lower activity than summer preparations, which is probably a reflection of dietary conditions. However, any particular preparation gave reasonably consistent results. The experiments described in the text were performed at different times over a period of 2 years, hence the difference in the extent of acetylation, since a number of liver preparations were made during that time.

Pigeon liver extracts

The extracts of acetone-dried powder were made up as required. The powder was ground in a mortar with an aqueous medium, the composition of which varied with the requirements of

the experiment. Usually the powder was ground with 0.15 M KF in the proportion of 60 mg. of powder per ml. of extraction medium. To perform the grinding a small quantity of the fluid was added to the powder to make a thick paste, after which the remaining fluid was added gradually with continuous grinding, the whole operation taking 5 minutes.

After centrifugation at 19000 g for 5 minutes the clear amber supernatant was pipetted directly into the manometer flasks, the amount of extract added being always equivalent to 60 mg. of powder.

It was found unnecessary to add Coenzyme A to these extracts, nor was cysteine needed to obtain a high rate of acetylation of sulfanilamide.

Rat Brain Mince

Whole rat brain was put through a small Latapie mincer. 3 grams of mince was suspended in a medium of the following composition: NAA 0.068M, KCl 0.15M, $MgCl_2$ 0.03M, to make a total volume of 6 ml. One ml. of the suspension was pipetted into the Warburg vessel after all other solutions had been added. Salt contents of vessels (final concentrations): NAA 0.015M; KCl 0.033M; $MgCl_2$ 0.006M; NaF 0.03M; sodium phosphate buffer 0.02M, pH 7.4,; choline chloride 0.001M; eserine sulphate 0.0004M. Other additions were as indicated in the text. 0.2 ml. of 20% KOH was added to the centre-well of the Warburg vessel, along with filter paper.

Rat Brain Homogenate

(a) For purposes of Ach synthesis a 25% homogenate was made using the Potter-Elvehjem type glass homogenizer. The whole brain, minus the medulla, was homogenized in a medium of the following composition: KCl 0.09M; NAA 0.04M; $MgCl_2$ 0.012M; sodium phosphate buffer 0.02M, pH 7.4. 1 ml. of homogenate was added to each Warburg vessel. Slight changes made in this medium from time to time will be indicated in the text, as will all other additions to the vessels.

(b) For purposes of sulfanilamide acetylation, in conjunction with pigeon liver extract:

1 g. of rat brain (cerebral hemispheres) was homogenized and made up to 5 ml. with a solution containing KCl 0.06M; NAA 0.04M; and $MgCl_2$ 0.04M. 0.5 ml. of the homogenate was added to the Warburg vessels.

The homogenate described was the one mainly used. However, any deviations in the manner of preparation will be indicated in the text.

Manometric Techniques.

Nearly all experiments were carried out in the conventional Warburg manometric apparatus. Gassing was completed during the equilibration period.

METHODS OF ANALYSIS

Acetylcholine Assay

Acetylcholine was measured by contractions of the leech muscle suspended in an aerated saline medium (22) of the following composition: NaCl 0.71%; KCl 0.032%; $CaCl_2$ 0.018%; $NaHCO_3$ 0.012%; glucose 0.077%.

All results are reported in terms of acetylcholine chloride, which was used as a standard. For the calibration of the leech muscle, 0.1 to 0.5 ml. of a solution of acetylcholine chloride (10 micrograms %) was added to the saline medium surrounding the muscle, and the contraction of the leech muscle measured on a kymograph. The leech muscle was considered to be calibrated when two consecutive additions of the same amount of acetylcholine gave the same contraction. The strength of the unknown solutions were adjusted so that additions of 0.1 to 0.5 ml. to the leech chamber would produce contractions of the same length as that produced by the additions of the standard solution. Eserine sulphate (0.1 mg.) was always added to the perfusion fluid in the intervals between contractions.

ATP and other phosphate esters were removed from the samples to be assayed by the following procedure:

At the conclusion of the experiment the 3.0 ml. contents of the Warburg vessel was poured into 2.0 ml. of NaH_2PO_4 (0.2M) and placed in a boiling water bath for 10 minutes. After cooling, 2.0 ml. of $BaCl_2$ (0.3M) was added and the pH adjusted to 7 with saturated $Ba(OH)_2$. The precipitate was centrifuged

down and the clear supernatant decanted, and the excess barium removed with Na_2SO_4 (0.5M).

If the solution was not assayed immediately, NaH_2PO_4 was added to bring the solutions to pH 5. No Ach is lost as a result of the above procedure.

A control was routinely run with every experiment to correct for the effect of substances on the leech muscle other than that of Ach synthesized during the experiment. At the beginning of an experiment a vessel containing the same components as those present in the experimental vessel was placed immediately in boiling water and treated as described above. The control value as shown by the leech assay was subtracted from that of the experimental vessel.

Lactic Acid Determination

Lactic acid determinations were made on the same samples that were assayed for Ach. The method used was that of Barker and Summerson (79) as modified by Umbreit et al (80)*.

Sulfanilamide Determination

Sulfanilamide determinations were made according to the method of Bratton and Marshall (81). All reagents and stock solutions were made up in the prescribed manner.

The principle of the method is as follows:

Sulfanilamide is diazotized with nitrous acid, ammonium

*The lactic acid estimations were made with the assistance of Miss Rosalind Cohen and Miss Anne Benson.

sulfamate is added to destroy excess nitrous acid, and the diazotized sulfanilamide is coupled with a suitable reagent for color development.

The most suitable color reagent has been found to be N-(1-naphthyl)-ethylenediamine dihydrochloride, which may be prepared in a state of high purity; its coupling is very rapid and uninfluenced by pH in the range 1 to 2. Furthermore, this range of pH has no effect on the color of the dye. The coupling of the colorless color reagent with the diazotized sulfanilamide produces a red color.

When a photoelectric colorimeter is used the peak of absorption is at 545 mu. However, in the work to be described the colors were assayed using a Fisher Electrophotometer at 525 mμ.

Trichloracetic acid was ideal as a protein precipitant since the blank given is so small that it can be neglected. The concentration of trichloracetic acid in the final mixture was 3%. The presence of acetylsulfanilamide was found to have no effect on the determination of sulfanilamide.

A standard curve for sulfanilamide determination

A stock solution of sulfanilamide containing 40 mg.% was prepared. From this, standard solutions of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mg% were prepared as follows:

The stock solution of 40 mg. % was diluted with water to 10 mg. %. Solutions of the above concentrations were made by

adding 0.5 ml., 1.0 ml., 1.5 ml., 2.0 ml., 2.5 ml., and 3.0 ml., respectively, of the diluted stock solution to 18 ml. of 15% trichloracetic acid and making each up to 100 ml. with water.

To 10 ml. of each of the prepared standard solutions was added 1 ml. of 0.1% $NaNO_2$ solution. 1 ml. of 0.5% ammonium sulfamate was added after the tubes had been mixed thoroughly and 3 minutes had elapsed from the time of adding the nitrite. 2 minutes is allowed for the excess nitrite to be destroyed, after which 1 ml. of a 0.1% solution of the color reagent is added. Color development is instantaneous. All determinations were done in duplicate. The colors were read against a reagent blank prepared by adding 1 ml. of water in place of the nitrite solution. Fig. 1 shows the standard "curve" obtained when Optical Density at 525 mu is plotted against sulfanilamide content.

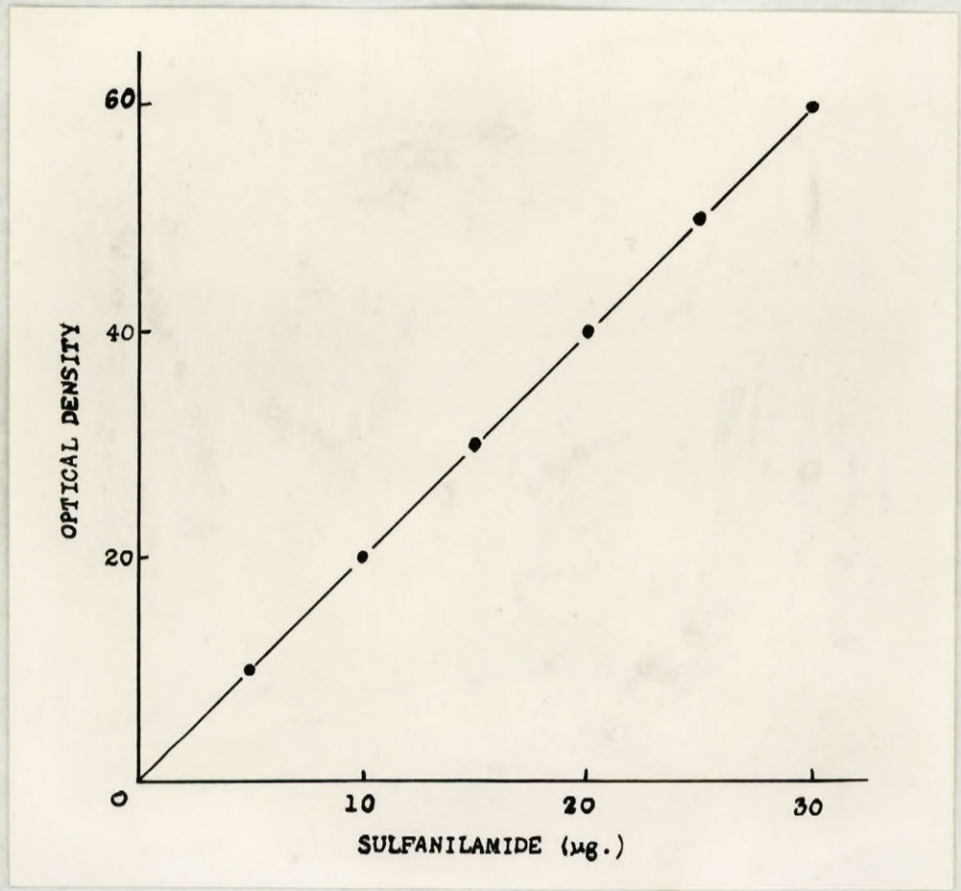


FIGURE 1.

A Standard Curve for Sulfanilamide Determination.
(as described in the text).

Estimation of Acetylsulfanilamide Content of Experimental Vessels

Acetylsulfanilamide was estimated by difference.

At the end of the incubation period 1 ml. of the Warburg vessel contents was pipetted into 14 ml. of 3.2% trichloracetic acid for protein precipitation. After centrifugation 5 or 10 ml. of the supernatant, depending on the initial concentration of sulfanilamide added to the vessel, was used for sulfanilamide determination. When rat brain homogenate was present in the reaction vessels the solid material was first spun down, and 1 ml. of supernatant added to the trichloracetic acid for protein precipitation.

With every experiment, a control containing the same components as the experimental vessels, was assayed immediately for sulfanilamide. The value obtained represented the sulfanilamide added to the experimental vessels. The value obtained on determining the sulfanilamide content of the experimental vessels after incubation, was subtracted from the value of the unincubated control to give the amount of sulfanilamide acetylated during the experiment. On occasions, a sample, the sulfanilamide content of which had been ascertained, was hydrolyzed by boiling with HCl for one hour and the sulfanilamide determined to ensure that conjugation alone was responsible for the disappearance of sulfanilamide during the experiment. It was found that complete recovery could always be obtained, which indicated that no sulfanilamide had been carried down by protein

precipitation. To illustrate the foregoing, during one experiment the unincubated control showed 103 ug. of sulfanilamide when assayed; the experimental vessel was estimated at 53 ug; the difference, 50 ug. was taken as the amount acetylated. A 10 ml. duplicate sample of supernatant of the experimental vessel was treated with 0.5 ml. of 4NHCl and heated in a boiling water bath for 1 hour to hydrolyze the acetylsulfanilamide. After cooling, the volume was adjusted to 10 ml. and the sample assayed for sulfanilamide. The duplicate samples gave a value of 105 ug. which was a recovery of 102% indicating that sulfanilamide disappearance during incubation and subsequent treatment could be accounted for by conjugation.

Complete recovery of sulfanilamide added to tissue preparations could always be obtained.

EXPERIMENTAL RESULTS

PYRUVATE DISMUTATION

The dismutation of pyruvate has been studied quite extensively in bacterial preparations (82,83,84). The E. coli preparations of Ochoa et al (84) require the following components for maximum activity: Orthophosphate, Mg^{++} or Mn^{++}, diphosphothiamine, Coenzyme A, DPN, a boiled extract of yeast, and lactic dehydrogenase. The dismutation reaction can be represented as follows:

$$\text{Pyruvate} + \text{phosphate} + \text{DPN} \longrightarrow \text{acetylphosphate} + CO_2 + \text{DPN.2H} \quad (1)$$

$$\text{Pyruvate} + \text{DPN.2H} \longrightarrow \text{lactate} + \text{DPN} \quad (2)$$

$$2\ \text{Pyruvate} + \text{phosphate} \longrightarrow \text{acetylphosphate} + CO_2 + \text{lactate} \quad (3)$$

Coenzyme A is presumably required for the formation of acetylphosphate. However, if oxalacetate is substituted for phosphate, citrate is formed instead of acetyl phosphate, the overall scheme for the reaction being:

$$\text{Pyruvate} + \text{DPN} + \text{CoA} \longrightarrow \text{Ac-CoA} + CO_2 + \text{DPN}_{red}$$

$$(-P)\ (+P) \qquad (-OAA)\ (+OAA) \quad (4)$$

$$\text{Ac-P} + \text{CoA} \qquad \text{citrate} + \text{CoA}$$

Apparently phosphate is required in the dismutation reaction to keep CoA in circulation.

Chantrenne and Lipmann (106) investigated the possibility that pyruvate might serve directly as acetyl donor in acetylation

reactions without the intermediate formation of acetate or acetylphosphate. Their extract of E. coli contained a formotransacetylase which catalyzed the following reaction:

Pyruvate (= acetyl formate) + CoA-enzyme ____ acetyl-CoA-enzyme + formate (5)

When the E. coli extract was mixed with the sulfanilamide acceptor enzyme from pigeon liver (55), acetylation of sulfanilamide occurred upon the addition of pyruvate. Only a trace of phosphate was present, which indicated the likelihood that acetylphosphate played no part in the reaction. The E. coli extract was unable to form acetylphosphate even in the presence of excess phosphate.

The investigation of pyruvate dismutation in animal tissues, as compared with that of bacteria, has been somewhat neglected. Krebs and Johnson (85), working with slices and minces, obtained evidence for the anaerobic dismutation of pyruvate by various tissues, and formulated the reaction as follows:

2 pyruvate + H_2O ——— acetate + CO_2 + lactate (6)

The highest rate of pyruvate removal was observed in testis; followed by brain, liver and kidney. The work of Krebs and Johnson was confirmed by others (83,84).

Acetylphosphate has never been identified as a reaction product of pyruvate dismutation in animal tissues, nor has it been reliably proven that acetylphosphate is formed in animal tissues under any conditions. Furthermore, attempts to utilize

acetylphosphate for acetylation purposes in animal tissue preparations have been unsuccessful (41) unless bacterial extracts were also added (55, 106). Therefore, Reaction 3 may only be applicable to the dismutation reaction with regard to bacteria, and quite a different mechanism of phosphate esterification may operate in animal tissues. Possibly, coincident with the oxidation of $DPNH_2$, phosphate is transferred to an adenylic compound resulting in ATP formation.

Pyruvate Dismutation and Acetylcholine Synthesis

Extracts of acetone-dried beef brain, when prepared in a specified manner (39) exhibit a rapid rate of anaerobic glycolysis which is manifested by a large production of lactic acid and CO_2 when carried out in a bicarbonate buffer. These glycolyzing extracts are capable of synthesizing acetylcholine, the amount of which is related to the rate and extent of glycolysis. It is probable that a portion of the pyruvate produced during glycolysis is diverted towards Ach synthesis, while the larger part is converted to lactic acid. It is also likely that a fraction of the lactic acid found on analysis arises through the dismutation of pyruvate, since, in an anaerobic system, pyruvate must dismute to give rise to the C_2 fragment, as no direct decarboxylation is known to occur in animal tissues. The dismutation of pyruvate, in the absence of glycolysis, should also give rise to the synthesis of acetylcholine providing the energy generated by dismutation is adequate to the needs of the system.

The Effect of Pyruvate on Glycolysis and Ach Synthesis

Pyruvic acid has been found to be strongly inhibitory to acetylcholine synthesis by cell-free extracts of mammalian central nervous system tissues (20, 29, 30, 35). Yet, pyruvic acid is formed in quantity from glucose when the latter undergoes glycolysis, and under these conditions Harpur and Quastel (39) demonstrated an active synthesis of acetylcholine by beef brain extracts. It is possible that the concentration of pyruvate required to inhibit Ach synthesis is never reached during glycolysis, and that the addition of pyruvate to a glycolyzing extract would have the customary inhibitory effect on Ach synthesis. It was decided that the point in question should be settled before proceeding further.

The methods of Harpur and Quastel (39) were used for the synthesis of Ach by cell-free extracts of beef brain.

The acetone dried brain powder (prepared as previously described) was extracted by grinding it in a solution of the following salts: nicotinamide, 0.08M; neutral cysteine, 0.03M; KCl, 0.09M; and $MgSO_4$, 0.012M. After centrifuging, the supernatant was filtered through glass wool, and 1 ml. of extract (equivalent to 60 mg. of powder) was added to each Warburg vessel. The vessels also contained, in addition to the substrates indicated in the tables, the following salts (final concentrations): $NaHCO_3$, 0.047M; eserine sulphate, 0.003M; choline chloride, 0.0008M; Na_4ATP, 0.0008M, sodium phosphate buffer, pH 7.5, 0.01M, in a total volume of 3 ml.

The experimental time was 1 hour, plus an equilibration period of 15 minutes, during which the vessels were gassed with 93% N_2, 7% CO_2 at 27°C.

It may be seen from the results shown in Table I that the addition of glucose to an extract of beef brain secures a high rate of anaerobic glycolysis and a greatly increased rate of acetylcholine (Ach) synthesis. The addition of a low concentration of pyruvate gave rise to a further increase in lactate formation and CO_2 output.

TABLE I

EFFECT OF PYRUVATE ON GLYCOLYSIS AND Ach SYNTHESIS BY BEEF BRAIN EXTRACT.

Exp.	Substrate	CO_2 µM.	Lactate µM.	Ach synthesis µg/g. powder
1	Nil	0	4.6	28
	Glucose 0.04M	36	53.0	105
	Pyruvate 0.005M	--	7.6	41
	Glucose 0.04M + pyruvate 0.005M	44	67.0	132
2	Nil	0	4.5	28
	Pyruvate 0.005M	--	6.7	37
	Glucose 0.04M	39	46.7	133
	Glucose 0.04M + pyruvate 0.005M	46	64.8	160
	Pyruvate 0.01M	4	8.8	64
	Glucose 0.04M + pyruvate 0.01M	47	66.5	160

Experimental conditions as shown on page 48.

Of greater interest are the facts that stimulation of glycolysis was accompanied by increased Ach synthesis, and that

pyruvate itself gave rise to a synthesis of Ach in the complete absence of glucose.

Pyruvate dismutation and Ach synthesis

Having established that pyruvate is not inhibitory to Ach formation under the given conditions, it was decided to investigate further the stimulation of acetylation by pyruvate alone, and to confirm what appeared to be the dismutation of pyruvate. The latter was thought to be of some significance, although no reference to its occurrence in cell-free extracts could be found in the literature.

TABLE II

ACETYLCHOLINE SYNTHESIS AND PYRUVATE DISMUTATION

	Control	Pyruvate 0.01M	Net
CO_2 (µM)	0.0	4.0	4.0
Lactate (µM)	4.3	8.0	3.7
Ach µg/g. powder	15	50	35

Brain extract (≡ 60 mg. of powder/vessel). Salt concentrations as shown on page 48. Pyruvate tipped from side arm at termination of 15 minute equilibration period.

The results shown in Table II indicate that the dismutation of pyruvate does, in fact, occur in cell-free extracts of beef brain. The net lactic acid found, and the

CO_2 output conform to the reaction as formulated by Krebs and Johnson (85):

$$2 \text{ Pyruvate} + H_2O \text{ ——— } \text{Acetate} + \text{Lactate} + CO_2 \qquad (6)$$

The above-named authors also observed that the presence of fumarate or malonate had no effect on the dismutation. These compounds were tested for possible effects on the beef brain system.

TABLE III

EFFECT OF FUMARATE AND MALONATE ON PYRUVATE DISMUTATION AND Ach SYNTHESIS*

Exp.	Additions	CO_2 uM	Ach synthesis ug/g. powder	Per cent inhibition of Acetylation
1	Nil	0.0	40	
	Pyruvate 0.01M	6.1	90	
	Malonate	0.0	40	0
	Pyruvate 0.01M + Malonate 0.01M	6.1	90	0
2	Nil	0.3	40	
	Pyruvate 0.01M	7.8	80	
	Fumarate 0.01M	0.0	15	62.5
	Pyruvate 0.01M + Fumarate 0.01M	8.2	24	70.0

* Brain extract = 60 mg/vessel. Other vessel contents as shown on page 48. DPN 0.00025M, ATP 0.001M. Pyruvate tipped from side arm at termination of 15 min. equilibration period.

The results shown in Table III (Exp. 1) indicate that the addition of malonate had no effect on the dismutation of

of pyruvate by the beef brain extract, since the output of CO_2 was unchanged in the presence of malonate; the rate of acetylcholine synthesis also remained the same. Although fumarate did not appreciably affect the CO_2 output (Table III, Exp. 2), it was responsible for a 70% inhibition of acetylcholine formation. Maleate, like malonate, had no effect on acetylcholine synthesis. (The inhibition by fumarate of both choline and sulfanilamide acetylation will be discussed in detail in a later section). It will be seen in Table III, that DPN was added in low concentration. Despite the use of nicotinamide in the preparation and extraction of the brain powder, it was observed that the addition of a low concentration of DPN to the brain extract stimulated the dismutation of pyruvate and the acetylation of choline.

Pyruvate dismutation and acetylation of sulfanilamide by pigeon liver extracts

Pigeon liver extract has proven to be an excellent medium for the investigation of the acetylation reaction. A high and constant rate of sulfanilamide acetylation can be obtained (Fig. 2) and the acetylated product is stable. In addition, sulfanilamide can be estimated by a sensitive chemical method (81), and acetylsulfanilamide determined by difference. Thus, the pigenn liver system offers definite advantages not to be found when one works with choline acetylation.

It was decided, therefore, to investigate the dismutation reaction in pigeon liver extracts and to ascertain to what extent the reaction can function in the acetylation of sulfanilamide.

Methods

Pigeon liver powder was prepared as described on page 34. The powder was extracted by rubbing it up with a solution of KF (0.15M), and $MgCl_2$ (0.033M). After centrifugation at 19000 g for 5 minutes, the clear supernatant was filtered through glass wool to remove surface substances and 1 ml. of extract (equivalent to 60 mg. of powder) was added to the Warburg flask. On some occasions, the $MgCl_2$ was omitted from the extraction fluid, and added, instead, directly to the Warburg vessel.

The extent of the dismutation reaction was determined

manometrically by measuring the formation of free CO_2 in a bicarbonate-phosphate buffer at pH 7.5. The vessels were gassed for 10 minutes with 93% N_2 and 7% CO_2; and a further equilibration period of 5 minutes was allowed to bring the contents to thermal equilibrium before the reaction was started by tipping sodium pyruvate from the side arm.

It was found, by a comparison of the amounts of sulfanilamide acetylated, that the reaction proceeded just as readily in the presence of air as under strictly anaerobic conditions. When an experiment was carried out with air as the gas phase, bicarbonate was omitted from the medium. However the reaction was still effectively an anaerobic one, since cell-free extracts of pigeon liver do not respire to an appreciable extent.

Rate of Synthesis of Acetylsulfanilamide

Although the rate of sulfanilamide acetylation, for the same amount of pigeon liver powder employed, varies to some extent from preparation to preparation, it was desirable to ascertain the variation of the rate of acetylation with time.

Warburg vessels were set up containing the following salts (final concentrations) in a total volume of 3 ml:

Sodium phosphate buffer, pH 7.4	0.02M
$NaHCO_3$	0.028M
Sodium acetate	0.02M

Na_4ATP	0.003M
Sulfanilamide	200 ug. (0.5 ml. of a 40 mg. % aqueous solution)

600 mg. of pigeon liver powder was extracted with 10 ml. of 0.15M KF, and 1 ml. of extract added to the manometer flask. 0.2 ml. of 0.045M ATP was placed in the side arm. After gassing for 10 minutes with 93% N_2 and 7% CO_2, and a thermal equilibration period of 5 minutes, the reaction was started by tipping the ATP into the main compartment of the flask. At periodic intervals of time the vessels were removed, and an aliquot of the contents assayed for sulfanilamide.

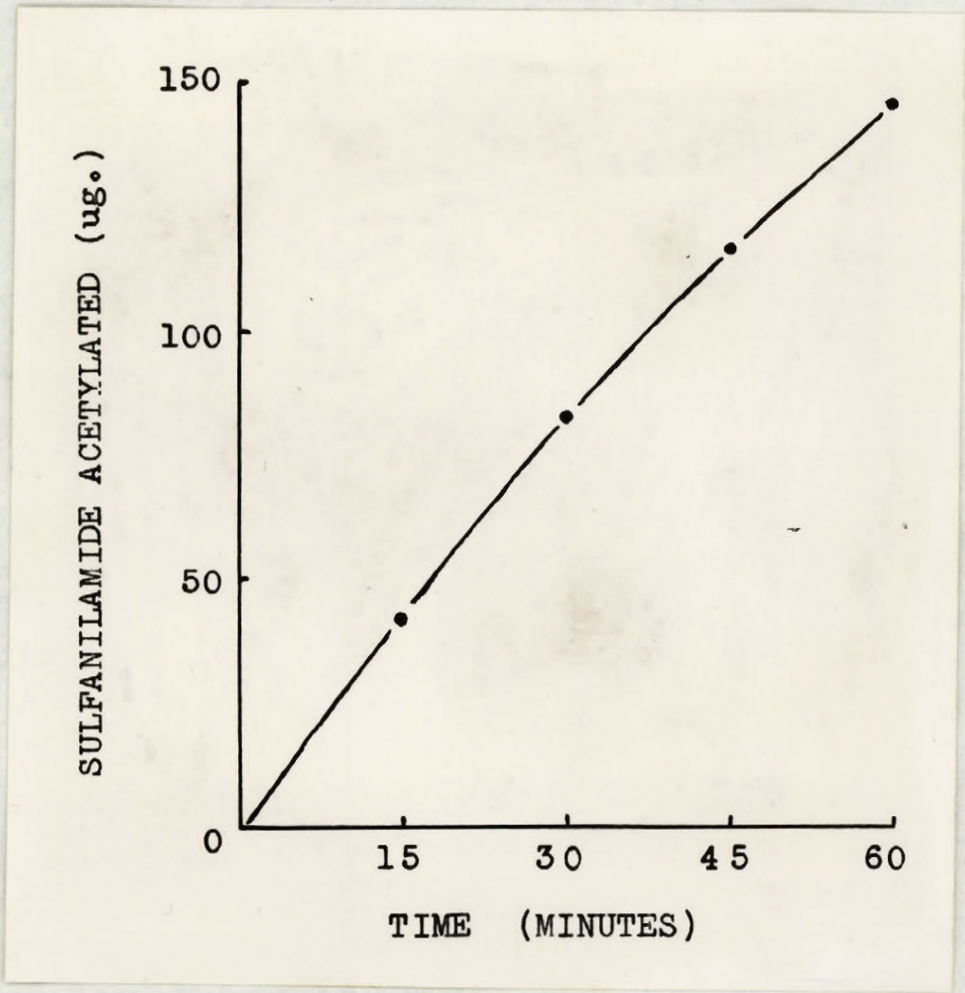


Figure 2.

Rate of Sulfanilamide Acetylation by Pigeon Liver Extract at 37° C.
(as described in the text).

A typical result shown in Fig. 2, indicates that the rate remains fairly constant over the whole period of time, in spite of the fact that 70% of the sulfanilamide had been acetylated at the end of 60 minutes. The failure of the rate to decline as the concentration of acetylsulfanilamide increases, implies the absence of an equilibrium point at which there is a detectable amount of sulfanilamide present. In fact, the acetylation of over 95% of the sulfanilamide initially added has been obtained (Table VIII, Exp. 5) in a period of 90 minutes.

Pyruvate Dismutation and Acetylation of Sulfanilamide

Pigeon liver extract when incubated in a bicarbonate-phosphate buffer at 37°C gives rise to but little CO_2 production and a low rate of acetylation of sulfanilamide. However, when pyruvate is added there is considerable CO_2 output and greatly increased acetylation, as can be seen from Table IV.

TABLE IV

PYRUVATE DISMUTATION AND ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT AT 37°C.

Exp.	Pyruvate conc.	CO_2 uM	Sulfanilamide added (ug)	Sulfanilamide acetylated (ug)
1	Nil	1.7	200	7
	0.02M	10.3	200	81
2	Nil	1.4	202	4
	0.02M	13.1	202	76
3	Nil	2.9	202	13
	0.01M	7.0	202	66

Pigeon liver extract: 1 ml. (≡ 60 mg. powder), prepared as described on page 53. Sodium phosphate buffer, pH 7.5 0.02M, $NaHCO_3$ 0.028M. Gassed for 10 min. with 93% N_2 and 7% CO_2. Reaction started by tipping pyruvate from the side arm. Duration: 90 minutes.

It is apparent that the dismutation of pyruvate has taken place, which accounts for the CO_2 generated and the sulfanilamide acetylated. Consequently, conditions are present in the extract whereby "active acetate" can be generated from pyruvate in the absence of added ATP. It may also be seen that the acetylation was not due to residual ATP in the crude extract since the addition of acetate alone to the extract produced no such increase in acetylation (Table V, Expt. 2; and Table VIII, Expt. 3 and 5).

It may also be noted that dismutation took place without the addition of DPN. This is due to the fact that the liver preparations contain small amounts of DPN even after extensive dialysis (193). Apparently all the necessary requirements for the dismutation of pyruvate are present, but not necessarily for the optimum rate of dismutation. This is indicated by the results shown in Table V, where it may be seen that a two-fold increase in acetylation was obtained by adding DPN to the system, while DPN had no appreciable effect on acetylation in the absence of pyruvate or in the presence of acetate. The probable role of DPN in the dismutation reaction will be discussed later.

TABLE V.

EFFECT OF DPN ON ACETYLATION OF SULFANILAMIDE

Exp.	Additions	Sulfanilamide added (ug)	Sulfanilamide acetylated (ug)
1	DPN 2.5 x 10^{-4}M	205	13
	Pyruvate 0.02M	205	66
	Pyruvate 0.02M + DPN 2.5 x 10^{-4}M	205	121
2	Acetate 0.02M	201	4
	Acetate 0.02M + DPN 2.5 x 10^{-4}M	201	7

Pigeon liver extract: 1 ml. (≡ 60 mg. powder) prepared as described on page 53.
Experimental conditions: See Table IV.

The Effect of Fluoride on Pyruvate Dismutation and Acetylation of Sulfanilamide

Although acetone powder extracts usually show little ATP-ase activity (34) fluoride was always included in the medium as a precautionary measure against losses in high-energy phosphate, unless its presence was undesirable for a particular reason. However, since magnesium is essential for pyruvate dismutation it was necessary to determine whether or not the usual concentration of fluoride used in preparing the pigeon liver extracts would inhibit the reaction. The inhibition of enolase by fluoride is a striking example of such an occurrence.

Therefore, the pyruvate dismutation and acetylating

activity of a sample of pigeon liver powder extracted with 0.15M KF was compared with a sample extracted with 0.15M KCl. Since 1 ml. of extract was added to each manometer flask in a total volume of 3 ml., the KF or KCl extract, as the case may be, was present in the flask at a concentration of 0.05M KF or KCl. It was also desirable to ascertain the effect of KF added directly to the vessel. The concentration of K^+ was maintained at a constant level, 0.1M in all vessels and only the fluoride concentration was varied.

The results of this experiment are shown in Table VI. It may be seen that the acetylation of sulfanilamide was greatest when the pigeon liver powder was extracted with 0.15M KF. Secondly, when the extraction was made with KCl the addition of fluoride had no appreciable effect on acetylation, although there was an increase in CO_2 output. Therefore, the best results were obtained when fluoride was present in the extraction medium rather than added directly to the manometer vessel. Thirdly, the addition of KF to a KF extract to make a total concentration of 0.1M fluoride inhibited the acetylation by 54%. The inhibition was probably due to excessive removal of magnesium from solution in the medium.

TABLE VI.

THE EFFECT OF FLUORIDE ON PYRUVATE DISMUTATION AND ACETYLATION OF SULFANILAMIDE

Additions	Liver powder extracted with 0.15M KCl		Liver powder extracted with 0.15M KF	
	Sulfanilamide acetylated ug.	CO_2 output µ litres	Sulfanilamide acetylated ug.	CO_2 output µ litres
KCl 0.05M	113	160	149	189
KF 0.05M	108	197	68	139

The Warburg vessels contained: 1 ml. of pigeon liver extract (≡ 60 mg. powder), Sulfanilamide 200 ug., 0.02M sodium phosphate buffer (pH 7.5), 0.028M $NaHCO_3$, 2.5×10^{-4}M DPN, 0.006M $MgCl_2$, Gassed for 10 minutes with 93% N_2 and 7% CO_2; 5 min. further equilibration, after which 0.2 ml. of 0.3M sodium pyruvate was tipped in to start the reaction. Total Volume: 3 ml.
Temperature: 37°C. Duration: 90 minutes.

The Effect of Orthophosphate on Pyruvate Dismutation and Acetylation of Sulfanilamide.

The fact that orthophosphate is required for pyruvate dismutation has been shown both for animal tissues (83,85,87) and bacteria (84,88,89).

This dependence on orthophosphate is well exemplified by the results shown in Table VII. It may be seen that in the absence of inorganic phosphate the CO_2 output and sulfanilamide acetylation were at a very low level, while the presence of a low concentration of phosphate (Exp. 2) gave rise to a large increase in both CO_2 production and acetylation of sulfanilamide.

TABLE VII.

EFFECT OF PHOSPHATE ON PYRUVATE DISMUTATION AND ACETYLATION BY PIGEON LIVER EXTRACT

Exp.	Additions	Experimental time (min.)	Sulfanilamide added (ug)	Sulfanilamide acetylated (ug)	CO_2 ul.
1	Pyruvate 0.02M	80	202	4	14
	Pyruvate + phosphate 0.02M		202	81	230
2	Pyruvate 0.02M	60	202	9	97
	Pyruvate 0.02M + phosphate 0.0067M		202	76	295

Pigeon liver extract: 1 ml. (≡ 60 mg. powder) prepared as described on page 53. Phosphate buffer, pH 7.5, as indicated in the table, $NaHCO_3$, 0.028M. Gassed with 7% CO_2 and 93% N_2.
Temperature: 37°C. Experimental Time: 90 minutes.

In Figure 3 are shown graphically the results of an experiment in which the phosphate concentration was varied. It may be seen that a low rate of CO_2 evolution occurred in the absence of added phosphate, which was probably due to traces of phosphate already present in the pigeon liver extract. However, the rate of CO_2 evolution increased sharply with increasing concentration of phosphate and reached a maximum at 0.0133M phosphate.

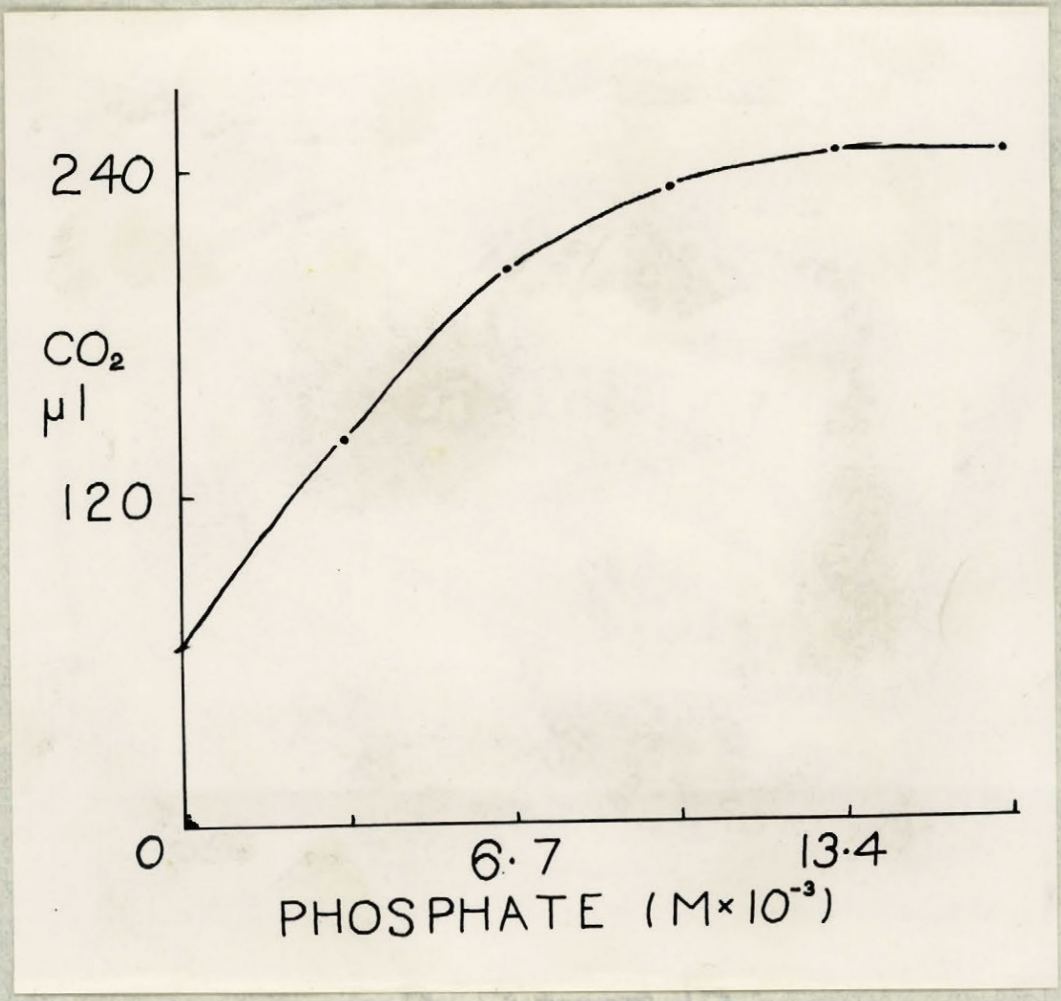


Figure 3.

The Effect of Phosphate Concentration on the Dismutation of Pyruvate by Pigeon Liver Extract. (Experimental Conditions: see Table VII).

The mechanism of pyruvate dismutation by the E. coli preparations of Ochoa, et al, as formulated in Reaction (4) relegates orthophosphate to a secondary role. Pyruvate gives rise directly, in the presence of CoA and DPN to acetyl--CoA while phosphate is required for the formation of acetyl-phosphate from acetyl-CoA with the simultaneous liberation of CoA. Phosphate thus serves to prevent the accumulation of

Ac – CoA and consequently, a deficiency of free CoA, without which dismutation cannot proceed. It will be noted that inorganic phosphate is not essential for dismutation since it can be replaced by oxolacetate. Presumably, if the sulfanilamide acetylating enzymes were present in these E. coli preparations, phosphate could also be replaced by sulfanilamide.

It becomes obvious that the formulation of the pyruvate dismutation reaction taking place in E. coli cannot apply to that of pigeon liver extract. Should Acetyl-CoA beformed directly from pyruvate and CoA without the intermediate action of inorganic phosphate, the acetylation of sulfanilamide would take place even in the absence of phosphate. However, the results shown in Table VII indicate that the acetylation of sulfanilamide is negligible in the absence of inorganic phosphate. It must be concluded from these results that the function of phosphate in the dismutation reaction by pigeon liver extracts differs from that obtaining in the E. coli preparations of Ochoa et al. It is apparent that in the former, phosphate is necessary for acetylation to take place under the given conditions, while in the latter, acetylation or citrate formation is entirely independent of the presence of phosphate.

The Effect of Adenylic Compounds on the Acetylation of Sulfanilamide in the Presence of Pyruvate and DPN.

Generally, a high rate of acetylation of sulfanilamide

was obtained by the addition of pyruvate and DPN to pigeon liver extracts when phosphate was also present. The addition of ATP to the dismutation system always increased the acetylation to a still higher level (Table VIII, Expt. 4 and 5), at times equalling that obtained by the use of acetate and ATP. It appeared, therefore, that the dismutation of pyruvate was adequate to provide a sufficient concentration of acetate to give rise to optimum synthesis of acetylsulfanilamide when ATP was also added.

On occasions a liver preparation was obtained which was capable of but a low rate of acetylation in the presence of pyruvate and DPN. One particular preparation had a very low activity as reflected in the synthesis of acetylsulfanilamide. However, upon the addition of acetate and ATP to this extract it was found that the acetylating activity was very high, which indicated that the CoA level and acetylating enzyme concentration were adequate. Furthermore, when pyruvate and DPN were added to the extract with a small quantity of ATP, the acetylation was increased from 31 ug. to 163 ug., an increase of 235% (Table VIII, Exp. 1). Therefore it was apparent that pyruvate was being broken down to provide the acetyl precursor, and that the lack of acetylation was probably due to a low level of adenylic compounds to act as phosphate acceptors during the dismutation.

TABLE VIII.

EFFECT OF ADENYLIC COMPOUNDS ON ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT.

Exp.	Additions	Sulfanilamide acetylated (ug)
1	Pyruvate 0.02M	22
	Pyruvate 0.02M + DPN*	31
	Pyruvate 0.02M + DPN + ATP 1.5×10^{-3}M	103
	Pyruvate 0.02M + DPN + ADP 1.5×10^{-3}M	81
	Pyruvate 0.02M + DPN + AMP 3×10^{-3}M	67
2	Pyruvate 0.02M + DPN	52
	Pyruvate 0.02M + DPN + ATP 0.003M	167
	Acetate 0.02M + ATP 0.003M	176
3	Acetate 0.02M	18
	Acetate 0.02M + AMP 0.003M	18
	Acetate 0.02M + ADP 0.003M	108
	Acetate 0.02M + AMP 0.003M + ADP 0.003M	86
	Acetate 0.02M + ATP 0.003M	185
4	Pyruvate 0.02M + DPN	153
	Pyruvate 0.02M + DPN + ATP 0.006M	185
5	Acetate 0.02M	09
	Acetate 0.02M + ATP 0.003M	193
	Pyruvate 0.02M	50
	Pyruvate 0.02M + DPN	99
	Pyruvate 0.02M + ATP 0.003M	124
	Pyruvate 0.02M + ATP 0.003M + DPN	180

Pigeon liver extract: 1 ml. = 60 mg. powder.
Temperature: 37°C.
Time; 90 minutes.
Experimental conditions: See Table IV.
Sodium pyruvate tipped from side arm after 15 min. gassing and equilibration.

*DPN con.: 2.5×10^{-4}.
Sulfanilamide added: 200 ug/vessel.

It will be seen from Table VIII, Exp. 3 that muscle adenylic acid (AMP) had no effect on the acetylation of sulfanilamide when added in the presence of acetate. However, AMP doubled the acetylation in the presence of pyruvate and DPN (Table VIII, Exp. 1).

To account for the stimulation of acetylation under the given conditions, it appeared that the addition of AMP must have increased the production of high-energy phosphate.

Barkulis and Lehninger (194) and Kielley and Kielley (91) have reported evidence which indicates that ADP, is the primary phosphate acceptor in oxidative phosphorylation. The latter have shown that complete phosphorylation of AMP can be obtained by pigeon liver mitochondrial preparations, dependent upon the presence of myokinase. However, to initiate the process, traces of ADP or ATP must be present from the start. Barkulis and Lehninger (194) working with rat liver particles obtained similar results. They stated however, that 0.03M fluoride inhibited rat liver myokinase but not purified muscle myokinase.

The events taking place in the pigeon liver extract can be interpreted in the light of the findings of the above authors. There are traces of ATP present in the extracts as indicated by the slight acetylation of sulfanilamide obtained upon the addition of acetate alone (Table VIII, Exp. 3 and 5). The presence and activity of myokinase are indicated by the

fact that 108 ug. of sulfanilamide was acetylated when ADP was added to the liver extract in the presence of acetate (Table VIII, Exp. 3). Since ADP cannot function as a phosphate donor in the absence of myokinase, the presence of the latter is confirmed. Furthermore, the high concentration of fluoride, 0.05M, present during the experiment did not inhibit myokinase activity. The fact that ADP and AMP, when present together gave rise to less acetylation of sulfanilamide than ADP alone indicated that AMP altered the position of equilibrium of the reaction catalyzed by myokinase so that the amount of ATP formed from ADP was decreased (Table VIII, Exp. 3).

Thus it can be seen that a necessary condition for acetylation to take place by virtue of the pyruvate dismutation reaction is the presence of adenylic compounds presumably to function as a phosphate acceptor during the dismutation. This conclusion is supported by the fact that AMP was unable to stimulate the acetylation of sulfanilamide when added in the presence of acetate, while the addition of AMP to the pigeon liver extract in the presence of pyruvate DPN and orthophosphate doubled the amount of acetylated sulfanilamide. The stimulation of the acetylation of sulfanilamide by AMP in the latter case is explanable only on the basis of indirect phosphorylation of AMP by the dismutation of pyruvate in the presence of myokinase.

The results shown in Table VIII, Exp. 4 indicate that

a fairly high level of phosphate esterification can be attained by the dismutation reaction. However, the fact that the addition of ATP stimulates the acetylation is an indication that the esterification of inorganic phosphate is a limiting factor. It will be noted that when DPN is omitted, the addition of ATP cannot give rise to maximum acetylation in the presence of pyruvate (Table VIII, Exp. 5). However, when DPN is added, there is sufficient breakdown of pyruvate in the system to give as high an acetylation when ATP is supplied to the system, as can be obtained with acetate and ATP.

Pyruvate Dismutation and Esterification of Phosphate

Assuming that ADP is the primary phosphate acceptor, at what stage in the dismutation reaction does the transfer of phosphate take place? A great deal of work has been done in recent years on the possible coupling of phosphorylation with the oxidation of reduced DPN. Lipmann, in 1946, concluded from thermodynamic considerations that the high-energy phosphate generated in aerobic phosphorylations is formed during the transfer of electrons from $DPNH_2$ to oxygen (92). Lehninger (93) has shown that esterification of phosphate is obtained during the oxidation of B-hydroxybutyrate to acetoacetate, that the primary dehydrogenation of substrate yields no high-energy phosphate, and also that the oxidation of reduced cytochrome c did not yield esterified phosphate under the same conditions as the oxidation of $DPNH_2$. Lehninger's experiments

provide indirect evidence that phosphate esterification may take place during the transfer of electrons between $DPNH_2$ and cytochrome c. The fact that ferricyanide oxidation of $DPNH_2$ gave rise to no esterification, does not invalidate the evidence, since the participation of cytochrome c in that instance may have been essential.

Aerobic phosphorylation is recognized as being closely associated with the mitochondrial fractions of the cell. It is possible that the same components of the cell are concerned with phosphorylations taking place under anaerobic conditions. In the presence of the mitochondrial components the oxidation of $DPNH_2$ which takes place during the dismutation of pyruvate may very well give rise to the same degree of phosphate esterification taking place during the oxidation of $DPNH_2$ under aerobic conditions.

Kaplan (95) states that factors, such as acetone, that disrupt mitochondria, also destroy the coupling of oxidation with phosphorylation. However, it is possible that components of the phosphorylation mechanism are present in extracts of acetone-dried preparations under certain conditions. It may be of some significance that 2,4-dinitrophenol has the same effect on the acetylation of sulfanilamide by a liver extract in the presence of pyruvate and DPN as it has on acetylations taking place under aerobic conditions.

Effect of 2,4-Dinitrophenol and Nitrourea

2,4-Dinitrophenol has been shown by Hotchkiss (97) to prevent the uptake of inorganic phosphate by respiring yeast, and by Loomis and Lipmann (96) to "uncouple" respiration from phosphorylation, i.e. to inhibit the formation of energy-rich bonds in a respiring system. It was of interest to compare the effect of nitrophenols on the phosphorylation involved in pyruvate dismutation under anaerobic conditions with the effect on a respiring system. Kaplan's statement (98) that "The anaerobic action of dinitrophenol appears to be dependent on the whole cell," adds interest to the effects obtained on acetone-powder extracts. The results of Exp. 1, Table IX indicate that while 2,4-DNP had negligible effect on acetylation per se as evidenced by the fact that practically no inhibition of acetylation was obtained in the presence of acetate and ATP, there was an inhibition of 62% in the pyruvate dismutation system. This inhibition is undoubtedly a reflection of the effect of 2,4-DNP on the phosphorylation mechanism. Exp. 2 (Table IX) shows the effect of 2,4-DNP on the acetylation of sulfanilamide, in the presence and absence of orthophosphate. The low rate of acetylation which took place in the absence of added phosphate was completely eliminated by 2,4-DNP, while in the presence of phosphate 4.5×10^{-4} M 2,4-DNP inhibited the acetylation by 31%. However, the inhibition exercised by the same concentration of 2,4-DNP on the acetylation of sulfanilamide under aerobic conditions,

TABLE IX.

EFFECT OF 2,4-DINITROPHENOL AND NITROUREA

ON THE ACETYLATION OF SULFANILAMIDE

Exp.	Additions	Sulfanilamide acetylated (ug)	Per Cent Inhibition of acetylation
1	Acetate 0.02M	04	-
	Acetate 0.02M + ATP 0.003M	153	-
	Acetate 0.02M + ATP 0.003M + 2,4-DNP 0.001M	139	9
	Pyruvate 0.02M	81	-
	Pyruvate 0.02M + 2,4-DNP 0.001M	31	62
2	Pyruvate 0.02M (no phosphate)	9	-
	Pyruvate 0.02M + 2,4-DNP 4.5 x 10^{-4}M (no phosphate)	0	-
	Pyruvate 0.02M + phosphate 6.7 x 10^{-3}M	76	0
	Pyruvate 0.02M + phosphate 6.7 x 10^{-3}M + 2,4-DNP 4.5 x 10^{-4}M	54	31
3	Acetate 0.02M + ATP 0.003M	144	-
	Acetate 0.02M + ATP 0.003M + Nitrourea 0.01M	119	17
	Pyruvate 0.02M	90	-
	Pyruvate 0.02M + Nitrourea 0.01M	54	40

Pigeon liver extract 1 ml. (≡ 60 mg. powder) /vessel. Temperature: 37°C. Time: 60 minutes + 15 minutes equilibration. $NaHCO_3$ 0.028M, sodium phosphate buffer 0.02M. Gassed with 93% N_2 and 7% CO_2. DPN 5 x 10^{-4}M added with pyruvate. Sulfanilamide 200 ug/vessel. ATP: neutralised with $NaHCO_3$. $MgCl_2$, 0.0067M. All substrates shown in the table added as the sodium salt, pH 7.4.

as will be shown later, was much greater than the effect of 2,4-DNP on the anaerobic system described.

Nitrourea, at a much higher concentration than 2,4-DNP shows the same type of action as the latter (Table IX, Exp. 3).

The Effect of Narcotics on the Pyruvate Dismuting System

2,4-DNP was shown to have no appreciable effect at high concentration on the acetylation of sulfanilamide in the presence of acetate and ATP, while at the same concentration, 0.001M, the acetylation of sulfanilamide by the pyruvate dismuting system was inhibited by 62%. It has been suggested (146) that narcotics, at low concentration, act in a manner similar to 2,4-DNP, i.e. to uncouple phosphorylation from respiration. Should this be so, one might expect narcotics to have an effect similar to that of 2,4-DNP on the pyruvate dismuting system.

However, the results shown in Table X indicate that the effects of 2,4-DNP and narcotics are quite dissimilar. Of the three narcotics tested, only hyoscine (scopalamine) exercised an effect resembling that of 2,4-DNP. In this case (Table X, Exp. 1) 0.005M hyoscine had no appreciable effect on the acetylation of sulfanilamide in the presence of acetate and ATP, while the rate of acetylation was inhibited by 38% when pyruvate was used. Furthermore, the inhibition by hyoscine was completely overcome by the addition of ATP, indicating the likelihood that hyoscine had depressed the generation of high-energy phosphate associated with the dismutation of pyruvate. It will be seen that in all experiments neither hyoscine nor chloretone at high concentration had any effect on acetylation when ATP was supplied (Exp. 1,2, and 4), although chloretone at the same concentration inhibited to the extent of 60 to 75% the acetylation of sulfanilamide taking place in a respiring

TABLE X

EFFECT OF CHLORETONE, NEMBUTAL AND HYOSCINE ON PYRUVATE DISMUTATION AND ACETYLATION OF SULFANILAMIDE

Exp.	Additions	S Sulfanilamide acetylated (ug)	Per cent inhibition of acetylation
1(a)	Acetate 0.02M + ATP 0.003M	153	-
	Acetate 0.02M + ATP 0.003M + Hyoscine 0.005M	148	-
(b)	Pyruvate 0.02M	74	-
	Pyruvate 0.02M + Hyoscine 0.005M	46	38
	Pyruvate 0.02M + ATP 0.003M	90	-
	Pyruvate 0.02M + ATP 0.003M + Hyoscine 0.005M	94	-
2	Acetate 0.02M + ATP 0.045M	193	-
	Acetate 0.02M + ATP 0.0045M + Chloretone 0.005M	195	-
3	Pyruvate 0.02M	121	-
	Pyruvate 0.02M + Chloretone 0.004M	108	10
*4	Pyruvate 0.02M + ATP 0.003M	137	-
	Pyruvate 0.02M + ATP 0.003M + Chloretone 0.004M	137	0
5	Pyruvate 0.02M	76	-
	Pyruvate 0.02M + Nembutal 0.001M	76	0

Pigeon liver extract: 1 ml (= 60 mg. powder)/vessel.
Time: 90 minutes + 15 minutes equilibration.
Temperature: 37°C.
$NaHCO_3$ 0.028M, sodium phosphate buffer 0.02M, pH 7.5 gassed with 93% N_2 and 7% CO_2. DPN 5×10^{-4}M was added wherever pyruvate was used. $MgCl_2$ 0.0067M; Sulfanilamide 200 ug. All substrates added as their sodium salts, pH 7.4. Hyoscine hydrobromide: neutralised with NaOH before use.

*Carried out in air. $NaHCO_3$ omitted, and the vessels not gassed.

system. Chloretone, at 0.004M concentration, inhibited acetylation by the pyruvate dismuting system to the extent of 10%, which is bordering on the limits of experimental error (Table X, Exp. 3). Nembutal, at 0.001M concentration, which inhibited the aerobic acetylation of sulfanilamide by 60% (Fig. 6), had no effect on the anaerobic acetylation (Table X, Exp. 5).

The Effect of Citrate on the Pyruvate Dismutation System

The activating effect of citrate on acetylcholine synthesis (20,21,27,28,29,30,38) has been well substantiated, but has been at the same time the object of some disagreement concerning the manner in which the activation is achieved (33, 24,21). (See "Historical Introduction", page 10).

In the present investigation it was confirmed that citrate had an activating effect on acetylcholine synthesis, by beef brain extract provided ATP was present (Table XIV). Similarily, citrate gave rise to increased acetylation of sulfanilamide by pigeon liver extract in the presence of ATP (Table XVI). It may be seen from Table XI, (Exp. 1) that no increase in acetylation was obtained when citrate alone was added to pigeon liver extract. However, the rate of sulfanilamide acetylation by pigeon liver extract in the presence of pyruvate was almost doubled when citrate was added (Table XI, Exp. 1 and 2). The stimulation of acetylation by citrate did not appear to be due to the inhibition of ATP-ase by the removal of divalent ions (23,24) since Oxalate (Exp. 2) had no effect (30); nor did

it appear that the activation of acetylation by citrate could be explained on the basis of the breakdown of citrate to give acetyl-CoA, since citrate when added alone had no effect on the rate of acetylation.

The citrate activation was similar to that obtained on the addition of DPN (Tables V and XI, Exp. 3), although no connection between the two phenomena was necessarily involved. It was rather surprising, however, that when citrate and DPN were added together the stimulating effect on acetylation was no greater than when one or the other was added alone (Table XI, Exp. 3.).

TABLE XI

THE EFFECT OF CITRATE ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT IN THE PRESENCE OF PYRUVATE

Exp.	Additions	Sulfanilamide added (ug)	Sulfanilamide acetylated (ug)
1	Nil	202	4
	Citrate 0.01M	202	5
	Pyruvate 0.02M	202	63
	Pyruvate 0.02M + citrate 0.01M	202	126
2	Pyruvate 0.02M	205	66
	Pyruvate 0.02M + citrate 0.02M	205	111
	Pyruvate 0.02M + oxalate 0.02M	205	57
3	Citrate 0.02M	201	9
	DPN 2.5×10^{-4}M	201	13
	Citrate 0.02M + DPN 2.5×10^{-4}M	201	4
	Pyruvate 0.02M	201	64
	Pyruvate 0.02M + DPN 2.5×10^{-4}M	201	121
	Pyruvate 0.02M + DPN 2.5×10^{-4}M + citrate 0.02M	201	117

Pigeon liver extract: 1 ml.(=60 mg. powder)/vessel. Sodium phosphate buffer 0.02M, pH 7.5, $NaHCO_3$ 0.028M. Gassed for 10 min. with 93% N_2 and 7% CO_2. $MgCl_2$ 0.005M. Pyruvate, citrate, oxalate, added as their sodium salts.
Temperature: 37°C. Experimental time: 90 minutes + 15 minutes equilibration.

Recently, Stern et al (206) have shown that the following reaction can take place in pigeon liver extract:

$$\text{citrate} + \text{CoA} + \text{DPN}_{red} \rightleftharpoons \text{l-malate} + \text{acetyl-CoA} + \text{DPN}_{ox} \quad (8)$$

Citrate breaks down to acetyl-CoA and oxalacetate; the removal of the latter by conversion to malate in the presence of malic dehydrogenase and $DPNH_2$ facilitates the breakdown of citrate. Equilibrium is reached when approximately 70% of the $DPNH_2$ is oxidized.

Reaction (8) provides an explanation for the activating effect of citrate on the acetylation of sulfanilamide in the presence of pyruvate. The reduced DPN required in Reaction (8) could be supplied from the first stage of the pyruvate dismutation reaction, i.e.

$$\text{Pyruvate} + \text{DPN} + \text{CoA} \longrightarrow \text{acetyl-CoA} + CO_2 + DPNH_2 \quad (9)$$

The result would be a coupling of Reaction (8) and (9) to give as the net result:

$$\text{Pyruvate} + \text{citrate} + \text{CoA} \xrightleftharpoons{\text{DPN}} \text{malate} + \text{acetyl CoA} + CO_2 \quad (10)$$

It was pointed out previously that the addition of citrate alone to the pigeon liver extract brought about no increase in the acetylation of sulfanilamide. The citrate stimulation of acetylation in the presence of pyruvate must then have been due to the fact that the $DPNH_2$ produced by Reaction (9) made possible the breakdown of citrate by means of Reaction (8) and hence increased the over-all formation of Acetyl-CoA.

It may be seen from Table XI (Expt. 3) that in the presence of added DPN citrate failed to stimulate the acetylation

of sulfanilamide, whether or not pyruvate was present. This result is understandable when one considers that the addition of a relatively high concentration of DPN in the oxidized form would drive Reaction (8) to the left, thus favouring the formation of citrate rather than the breakdown. Furthermore, under these conditions, when pyruvate is present, the additional effect of the lactic dehydrogenase system (Reaction 13) would tend to prevent the $DPNH_2$ concentration from reaching the point where citrate breakdown could occur. Hence, in this case citrate would have no stimulating effect on the rate of sulfanilamide acetylation. An alternative explanation for the phenomenon may be that Coenzyme A is the rate limiting factor since it takes part in both Reactions (8) and (9), in which case the addition of citrate in the presence of DPN would have no effect on the rate of acetylation. The addition of Coenzyme A to the system would have settled this point. However, it had been found that the pigeon liver extracts contained optimum amounts of Coenzyme A for acetylation purposes since the rate of acetylation could not be increased by the addition of boiled tissue extracts.

The Effect of Oxalacetate and Fumarate on the Acetylation of Sulfanilamide by Pigeon Liver Extract in the Presence of Pyruvate and DPN

Oxalacetate has been found to inhibit acetylcholine synthesis by brain extracts (30) and the acetylation of aromatic amines by pigeon liver extracts (63).

The large inhibition exercised by oxalacetate on sulfanilamide acetylation is shown in Table XII (Exp. 1). It is now known that this inhibition is due to competition by oxalacetate for Acetyl-CoA, thus decreasing the formation of acetylsulfanilamide (99).

TABLE XII

EFFECT OF OXALACETATE AND FUMARATE ON PYRUVATE DISMUTATION AND ACETYLATION OF SULFANILAMIDE

Exp.	Additions	Sulfanilamide acetylated ug.	Per cent inhibition of acetylation
1	Pyruvate 0.02M + DPN 2.5 x 10^{-4}M	78	-
	Pyruvate 0.02M + DPN 2.5 x 10^{-4}M + Oxalacetic 0.005M	26	67
	Pyruvate 0.02M + DPN 2.5 x 10^{-4}M + ATP 0.003M	137	-
	Pyruvate 0.02M + DPN 2.5 x 10^{-4}M + ATP 0.003M + Oxalacetate 0.005M	65	53
2	Pyruvate 0.02M + DPN 5 x 10^{-4}M	141	
	Pyruvate 0.02M + DPN 5 x 10^{-4}M + fumarate 0.02M	45	68
3	Acetate 0.02M + DPN 5 x 10^{-4}M + ATP 0.003M	137	-
	Acetate 0.02M + DPN 5 x 10^{-4}M + ATP 0.003M + fumarate 0.02M	47	65

Pigeon liver extract: 1 ml (≡ 60 mg. powder)/vessel.
Temperature: 37°C. Time: 90 minutes + 15 minutes equilibration.
Sodium phosphate buffer, 0.02M, pH 7.5. $NaHCO_3$ 0.028M; $MgCl_2$ 0.005M; Sulfanilamide 202 ug. Gassed for 10 minutes with 93% N_2 and 7% CO_2.
All substrates added as their sodium salts.
DPN was added in Exp. 3 to facilitate the oxidation of malate to oxalacetate.

The inhibition of acetylation by fumarate is shown in Exp. 2 (Table XII). The extent of the inhibition, which is similar to that shown by oxalacetate, indicates that fumarate can go readily to oxalacetate in pigeon liver extracts. The relevant equations are:

$$\text{Fumarate} + H_2O \xrightleftharpoons{\text{fumarase}} \text{malate} \qquad (11)$$

$$\text{Malate} + \text{DPN}_{ox} \xrightleftharpoons{\text{malic dehydrogenase}} \text{Oxalacetate} + \text{DPN}_{red} \qquad (12)$$

$$\text{DPN}_{red} + \text{Pyruvate} \xrightleftharpoons{\text{Lactic dehydrogenase}} \text{DPN}_{ox} + \text{lactate} \qquad (13)$$

It will be observed (Table XII, Exp. 3) that fumarate gave rise to the same degree of inhibition in the presence of acetate, DPN, and ATP as it did in the presence of pyruvate. In this case the pyruvate required for the oxidation of $DPNH_2$ may have been supplied in sufficient quantity by decarboxylation of oxalacetate.

The inhibition of acetylation reactions by fumarate and oxalacetate will be discussed in greater detail in the following section.

Summary

1. Contrary to the inhibitory effect of pyruvate on acetylcholine synthesis under other conditions, it has been shown that the addition of pyruvate to a beef brain extract in the presence of glucose does not inhibit the acetylation of choline. In fact, the addition of pyruvate stimulates both glycolysis and acetylcholine synthesis.

2. The addition of pyruvate to a beef brain extract results in a dismutation reaction the extent of which may be measured under anaerobic conditions by following the evolution of CO_2 and/or the formation of lactate.

3. The dismutation of pyruvate by beef brain extracts is associated with an increased rate of acetylcholine synthesis.

4. The addition of fumarate has no effect on the dismutation of pyruvate, but inhibits acetylcholine synthesis markedly.

5. The dismutation of pyruvate taking place in an extract of pigeon liver is associated with a large increase in the rate of sulfanilamide acetylation.

6. Although sulfanilamide acetylation may be obtained by the addition of pyruvate to pigeon liver extract the rate of acetylation is greatly increased by the addition of DPN, which is essential for the dismutation of pyruvate.

7. The dismutation of pyruvate by pigeon liver extracts does not occur to an appreciable extent in the absence of added orthophosphate. Furthermore, sulfanilamide cannot replace

orthophosphate in the reaction, which is indicated by the fact that the acetylation of sulfanilamide is negligible in the absence of phosphate.

8. In the presence of pyruvate and DPN, the addition of ATP, ADP, and AMP to a pigeon liver extract results in a large increase in the rate of sulfanilamide acetylation.

9. Although ATP and ADP are effective in stimulating the acetylation of sulfanilamide in the presence of acetate, AMP has no effect.

10. The fact that sulfanilamide acetylation can be obtained with acetate and ADP indicates the presence of myokinase in pigeon liver extract.

11. 2,4-Dinitrophenol, nitrourea, and to a lesser extent, hyoscine, inhibit the acetylation of sulfanilamide which is associated with pyruvate dismutation, but do not inhibit acetylation when acetate and ATP are used as acetylating agents. On the other hand, narcotics in general have no effect on the dismutation of pyruvate nor do they inhibit the anaerobic acetylation of sulfanilamide.

12. The rate of sulfanilamide acetylation by pigeon liver extract in the presence of pyruvate is approximately doubled when citrate or DPN is added. The addition of citrate and DPN together secures no further increase in the rate of acetylation.

13. Oxalacetate, like fumarate, markedly inhibits the rate of sulfanilamide acetylation by pigeon liver extract in the presence of pyruvate and DPN.

THE INHIBITION OF ACETYLATION BY DICARBOXYLIC ACIDS.

∝-Keto acids (pyruvic, oxalacetic, and -Keto-glutaric) in low concentrations inhibit the synthesis of Ach by cell-free extracts (20,29,30,35). Various explanations have been offered as to the mechanism of inhibition. Nachmansohn and John (20) taking into consideration the specific effect of l(+)-glutamic acid in reactivating the dialyzed enzyme extract suggested that the ∝-keto acids block the acetylating system by a competitive reaction between -CO--COOH and -CH.NH_2.COOH groups of the keto acid and glutamic acid, respectively; furthermore they thought it improbable that the inhibition was due to an effect on the -SH groups of choline acetylase. However, Nachmansohn (188) also points out that the degree of inhibition varies with the purity of the enzyme preparation. This would indicate that the inhibition is related to substances or enzymes removed in the course of purification of the enzyme preparation, rather than to a direct effect on the choline acetylating system per se.

The Effect of Fumarate, Malate, and Oxalacetate on Ach Synthesis by Cell-Free Extracts

Methods

The methods of Harpur and Quastel (39) were used for the synthesis of Ach by cell-free extracts of beef brain. The preparation of acetone-dried brain powder is described under "Experimental Methods and Procedures", page 33. For details as

to the experimental conditions for Ach synthesis by cell-free extracts of beef brain see page 48.

The Inhibition of Acetylcholine Synthesis by Oxalacetate

Extracts of beef brain, which are capable of a high rate of glycolysis produce but little Ach in the absence of added substrate (Table XIII). The addition of glucose gives rise to a greatly increased Ach synthesis which is related to the high rate of glycolysis as manifested by the lactate and CO_2 produced. It will be seen from Table XIII that there is a slight discrepancy between the lactic acid and CO_2 values. This is due to the fact that no correction was made for the lactic acid produced during the 15 minute equilibration period, whereas the measurement of CO_2 output was begun after that period. Since in the glycolytic sequence there is a net gain of ATP it may be expected that the increased Ach synthesis is a direct result of the breakdown of glucose to provide both the acetyl precursor and the ATP necessary for Ach synthesis to take place.

The addition of Oxalacetate at a concentration of 0.005M (15 uM) inhibited the acetylation of choline by 63% while at the same time increasing slightly the lactate and CO_2 production (Table XIII, Exp. 1). The latter probably indicates decarboxylation of some oxalacetate to pyruvate and CO_2, and further reduction of pyruvate to lactate. It will be recalled that pyruvate (Table I) under the same conditions did not inhibit the acetylation of choline, but did enhance, in a

TABLE XIII

THE EFFECT OF FUMARATE, MALATE, AND OXALACETATE ON GLYCOLYSIS AND ACH SYNTHESIS BY BEEF BRAIN EXTRACTS AT 37°C.

Exp.	Additions	Lactate uM	CO_2 output uM	Ach synthesis ug/g powder
1	Nil	4.6	--	28
	Glucose 0.04M	53.0	36	105
	Oxalacetate 0.005M	7.3	--	17
	Glucose 0.04M + Oxalacetate 0.005M	57.0	43	39
2	Nil	9.3	--	48
	Glucose 0.04M	58.4	31.5	119
	Fumarate 0.01M	9.3	--	20
	Glucose 0.04M + Fumarate 0.01M	54.7	28.5	35
3	Nil	--	--	43
	Glucose 0.03M	--	46	100
	Malate 0.01M	--	--	10
	Glucose 0.03M + Malate 0.01M	--	41	43
4	Nil	--	--	24
	Fumarate 0.01M	--	--	10
	Glucose 0.01M	--	38	80
	Glucose 0.01M + Fumarate 0.01M	--	39	20
	Maleate 0.01M	--	0.5	20
	Glucose 0.01M + Maleate 0.01M	--	38	80

Bicarbonate-phosphate buffer, pH 7.5. Gassed with 93% N_2 and 7% CO_2. Experimental conditions as described on page 48.

manner similar to oxalacetate, the output of CO_2 and the formation of lactate. It in unlikely that oxalacetate exercised an inhibition of acetylation by virtue of the α-keto group, since pyruvate which also contains the same grouping did not inhibit acetylation under the same conditions (Table I).

Inhibition of the Acetylation of Choline by Fumarate and Malate

Dicarboxylic acids (including fumarate and malate) have been found to be ineffective in the reactivation of dialyzed extracts (20). However, the effects of fumarate and malate on active brain extracts have been given scant attention (18).

It was in the hope of gaining some insight into the inhibition of the acetylation of choline caused by oxalacetate that fumarate and malate were first tested for possible affects on acetylation, since they are precursors of oxalacetate in the tricarboxylic acid cycle. It was found that both malate and fumarate inhibited the acetylation of choline to the same extent as did oxalacetate, (Table XIII, Exp. 2 and 3), but the effect of fumarate on lactate production and CO_2 output was the opposite to that shown by oxalacetate, although the effect one way or the other was quite small.

The lack of inhibition by maleate, the cis-isomer of fumarate, (Table XIII, Exp. 4) indicated the likelihood that fumarate and malate were not inhibitory to Ach synthesis by virtue of the carboxyl groups which they possess; therefore, the probability was great that they were oxidized to oxalacetate.

The Effect of Fumarate on Ach Synthesis in the Presence of Citrate

The stimulating effect of citrate on Ach synthesis

is shown in Table XIV (Exp. 1). (It has been shown by Lipton and Barron (21) that citrate breaks down to provide "active" acetate for acetylation purposes). The addition of fumarate to this system resulted in a much greater inhibition of acetylation than was the case when fumarate was added to the glycolyzing system shown in Table XIII. It appeared that fumarate prevented the breakdown of citrate, since the acetylation was reduced to the control value. This indicated the possibility of the accumulation of oxalacetate from fumarate, thus shifting the equilibrium in favour of citrate synthesis rather than breakdown.

It was expected that the addition of semicarbazide to the fumarate inhibited system would relieve the inhibition by trapping the oxalacetate formed (Table XIV, Exp. 2). Such was not the case. However, semicarbazide increased the synthesis of Ach in the presence of citrate by 26%, which confirmed the observation of Lipton and Barron (21) that semicarbazide stimulates Ach synthesis, by fixing the oxalacetate which accumulates as the result of the breakdown of citrate, thus propitiating the formation of "active acetate" for acetylation purposes.

The work on Ach synthesis by beef brain extracts indicated that fumarate and malate were not in themselves inhibitory, but that oxalacetate formed from these compounds was responsible for the inhibition. However, direct evidence was necessary to show the manner in which the malate arising from

TABLE XIV

THE FUMARATE INHIBITION OF ACH SYNTHESIS

BY BEEF BRAIN EXTRACT IN THE PRESENCE OF CITRATE
(at 37°C).

Exp.	Additions	Ach Synthesis ug/g powder	Per cent inhibition by Fumarate
1	Nil	26	--
	Citrate 0.01M	140	--
	Fumarate 0.01M	15	42
	Citrate 0.01M + Fumarate 0.01M	26	81
2	Nil	45	--
	Citrate 0.01M	116	--
	Fumarate 0.01M	24	47
	Citrate 0.01M + Fumarate 0.01M	52	55
	Semi-Carbazide 0.02M	47	
	Citrate 0.01M + Semi-Carbazide 0.02M	157	
	Citrate 0.01M + Fumarate 0.01M + Semi-Carbazide 0.02M	52	55

Experimental Conditions: 1 ml. of brain extract prepared as described on page 33 . $NaHCO_3$ 0.037M, eserine sulphate 0.003M, choline chloride 0.0008M, Na_4ATP 0.002M, NaF 0.03M, sodium phosphate buffer, pH 7.5, 0.01M.
Total Volume: 3 ml. Gassed with 93% N_2 and 7% CO_2.
Time: 1 hour plus 15 minutes equilibration.

fumarate could be anaerobically oxidized to oxalacetate, and also to provide proof that fumarate and malate were not inhibitors *per se* of acetylation. The sulfanilamide acetylating system of pigeon liver was employed for this phase of the work.

Fumarate Inhibition of Sulfanilamide Acetylation

Pigeon liver acetone powder, prepared as described on page 34 was extracted by grinding it for 5 minutes in a

a mortar with the following solution: KCl 0.09M, $MgCl_2$ 0.012M, and NaF 0.09M. Sufficient extraction solution was added initially to the powder to make a smooth thick paste after which the solution was added in small quantities with continuous grinding until the requisite amount of extraction solution had been added. After centrifugation at 19000 g for 5 minutes, 1 ml. of the supernatant, equivalent to 60 mg. of powder, was pipetted directly into each manometer flask. Other additions to the flasks are shown in the tables.

TABLE XV

FUMARATE INHIBITION OF SULFANILAMIDE ACETYLATION BY PIGEON LIVER EXTRACT AT 37°C

Exp.	Additions	Sulfanilamide added (ug)	Sulfanilamide acetylated (ug)	Per cent inhibition by fumarate
1	Nil	199	18	
	Acetate 0.013M	199	111	--
	Fumarate 0.01M	199	7	61
	Acetate 0.013M + fumarate 0.01M	199	63	43
	Acetate 0.013M	100	78	--
	Acetate 0.013M + fumarate 0.01M	100	42	46
2	Acetate 0.013M	101	66	--
	Acetate 0.013M + fumarate 0.02M	101	29	56

Media: 1 ml. of pigeon liver extract (= 60 mg. powder) $NaHCO_3$ 0.028M, ATP 0.002M. Gassed with 93% N_2 and 7% CO_2.
Time: 1 hour.

In the presence of acetate and ATP, fumarate inhibited the acetylation of sulfanilamide by 43% (Table XV, Exp. 1), and by reducing the concentration of sulfanilamide the inhibition of acetylation by fumarate was increased to a slight extent, but not significantly. However, by increasing the fumarate concentration from 0.01M to 0.02M the inhibition of acetylation was increased to 56% (Exp. 2). The fact that the rate of acetylation could be reduced by increasing the concentration of fumarate relative to sulfanilamide indicated the probability of substrate competition for "active acetate".

The Effect of Fumarase Inhibitors

The first step in the formation of oxalacetate from fumarate involves the addition of a molecule of water to the latter to give rise to malate. Quastel (189) has shown that the acidic dye, Congo Red, at one part in 5000 inhibits fumarase activity of a cellular extract of E. coli by 97%. Therefore, the addition of Congo Red to the pigeon liver extract might be expected to prevent the formation of oxalacetate from fumarate by blocking the transformation at the fumarase stage, thus effectively overcoming the inhibition of acetylation caused by the addition of fumarate. However, Congo Red would not be expected to relieve the inhibition of acetylation caused by malate or oxalacetate. Nertheless, a relief of the fumarate inhibition by Congo Red would constitute proof that fumarate per se was inhibitory to the acetylation process.

The effects of the addition of Congo Red are shown in

Table XVI, Exp. 1 and 2. It may be seen that Congo Red had no effect on the acetylation of sulfanilamide (Exp. 1). However when added in the presence of fumarate, malate, or oxalacetate Congo Red increased the inhibition of acetylation by 14 to 20%. No explanation is offered for this phenomenon. It should be mentioned that Congo Red did not interfere with the determination

TABLE XVI

THE EFFECT OF CONGO RED (CR) ON THE INHIBITION OF SULFANILAMIDE ACETYLATION BY FUMARATE, MALATE, AND OXALACETATE AT 37°C

Exp.	Additions	Sulfanilamide acetylated (ug)	Per cent Inhibition
1	Acetate 0.02M	64	
	Acetate 0.02M + Congo Red	64	
	Acetate 0.02M + Oxalacetate 0.02M	25	61
	Acetate 0.02M + Oxalacetate 0.02M + CR	16	75
2	Acetate 0.02M	70	
	Acetate 0.02M + Fumarate 0.02M	32	54
	Acetate 0.02M + Fumarate 0.02M + CR	18	74
	Acetate 0.02M + Malate 0.02M	41	41
	Acetate 0.02M + Fumarate 0.02M + CR	27	61
	Acetate 0.02M + Malate + Fumarate 0.01M	37	47

Media: 1 ml. of pigeon liver extract (≡ 60 mg. powder), Sulfanilamide, 100 ug. $NaHCO_3$ 0.028M; ATP 0.002M; Congo Red $\frac{1}{4500}$. Gassed with 93% N_2 and 7% CO_2.

Time: 1 hour.

of sulfanilamide as it was carried down completely by the trichloracetic acid-precipitated proteins, leaving a clear, colorless supernatant.

Bayer-205 (suramin) was also tried as a fumarase inhibitor (189). At a concentration of 3.5×10^{-4} M, Bayer-205 inhibited completely the acetylation of sulfanilamide.

It was noticed that fumarate, in a number of experiments, gave rise to a greater inhibition than was the case with malate. This suggested the possibility that fumarate was necessary as a hydrogen acceptor for the oxidation of malate to oxalacetate, which would explain the greater formation of oxalacetate from fumarate than from malate. Although an equilibrium exists between fumarate and malate, in which case there would always be some fumarate present when malate alone was added, the equilibrium state is heavily in favour of malate. It would be expected, if fumarate acts as a hydrogen acceptor for malate oxidation, that the addition of fumarate would enhance the malate effect. However, when fumarate and malate were added together (Table XVI, Exp. 2) the inhibition of acetylation was an average of that obtaining when each was present alone.

As a further check on the possibility that fumarate might act as a hydrogen acceptor for malate oxidation, it was decided to assay the flask contents for the presence of succinic acid. An active succinoxidase preparation was obtained from pigeon breast muscle (190) for this purpose. How-

ever, no indication of succinic acid formation was obtained. Furthermore, assay of the pigeon liver extract for succinic dehydrogenase by means of the ferricyanide technique (172) gave negative results. In view of the above data it appeared that, provided fumarate and malate were causing the inhibition by means of the formation of oxalacetate, a compound other than fumarate must act as hydrogen acceptor for the oxidation of malate.

The Effect of Cyanide on the Fumarate Inhibition of Acetylation

Cyanide and semicarbazide form with oxalacetic acid the cyanhydrin and semicarbazone, respectively, of that α-keto-acid (191). Therefore, the presence of one of the above keto-acid fixatives should effectively trap the oxalacetate arising from fumarate and thus relieve the inhibition of acetylation.

Semi-carbazide was first tried in connection with Ach synthesis (Table XIV) where it was found that at the concentration used, 0.02M, the inhibition by fumarate was not relieved, but that semicarbazide stimulated acetylation in the presence of citrate. Semicarbazide was found to be unsuitable as a trapping agent for oxalacetate when sulfanilamide acetylation was involved since it inhibited the latter process.

It has been shown by Green and Williamson (191) that cyanide is about three times as efficient as semicarbazide as a fixative for oxalacetate. At the same time, cyanide does not affect the acetylation process (Table XVII, Exp. 1).

Methods

The pigeon liver extract was prepared by grinding the powder (prepared as described under "Experimental Methods and Procedures") with 0.15M KF for 5 minutes. 1 ml. of KF was used per 60 mg. of powder. After centrifugation at 19000 g the clear supernatant was pipetted directly into the manometer flasks. It will be noted that it was unnecessary to add Coenzyme A or cysteine to these preparations in order to obtain a high rate of acetylation. Thus, in Exp. 1 and 3 of Table XVII, over 80% of the added sulfanilamide was acetylated in a period of 75 minutes. It has been found throughout this work that the crude extracts of pigeon liver have always contained sufficient Coenzyme A for acetylation purposes, and increased activity was not obtained by increasing the concentration of CoA.

Since NaCN was used in high concentrations, the sodium ion concentration in all vessels was equalized by the addition of NaCl where required. The incubation was carried out for a period of 90 minutes at 37°C in a bicarbonate-phosphate buffer composed of $NaHCO_3$ 0.028M and sodium phosphate, pH 7.5, 0.02M. Other additions to the manometer flasks are shown in the tables. All concentrations designated are final, in a total volume of 3 ml.

Results

It may be seen from Table XVII, Exp. 1 and 3, that fumarate exercised its characteristic inhibition of acetylation

although the effect was not as great as usually observed. However, the addition of cyanide decreased the inhibition by 57%, indicating that the inhibition of acetylation was in all probability due to the formation of oxalacetate and not to

TABLE XVII

THE EFFECT OF CYANIDE ON THE INHIBITION OF ACETYLATION BY FUMARATE (at 37°C)

Exp.	Additions	Sulfanilamide acetylated ug.	Per Cent Inhibition of Acetylation
1	Acetate 0.02M	162	--
	Acetate 0.02M + NaCN* 0.067M	162	--
	Acetate 0.02M + Fumarate 0.013M	117	28
	Acetate 0.02M + Fumarate 0.013M + NaCN 0.067M	142	12
2	Acetate 0.02M	158	
	Acetate 0.02M + NaCN 0.1M	155	
	Acetate 0.02M + Fumarate 0.02M	112	29
	Acetate 0.02M + Fumarate 0.02M + NaCN 0.1M	135	14
3	Acetate 0.02M	83	
	Acetate 0.02M + Fumarate 0.02M	67	19
	Acetate 0.02M + Fumarate 0.02M + NaCN 0.1M	79	5
	Acetate 0.02M + DPN 0.0003M	81	
	Acetate 0.02M + DPN 0.0003M + Fumarate 0.02M	56	31
	Acetate 0.02M + DPN 0.0003M + Fumarate 0.02M + NaCN 0.1M	76	6

Media: Bicarbonate-phosphate buffer pH 7.5, 1 ml. of pigeon liver extract (= 60 mg. powder) as described in the text.
Sulfanilamide: Exp. 1 and 2, 203 ug
Exp. 3, 101 ug
$MgCl_2$ 0.004M. Gassed with 93% N_2 and 7% CO_2.
Time: 60 minutes plus 15 minutes equilibration.

* NaCN was neutralised with HCl before being added to the manometer flasks.

fumarate *per se*. The effect was still more apparent in Exp. 3 (Table XVII) where the inhibition by fumarate was enhanced by the addition of DPN to facilitate the oxidation of malate to oxalacetate. Although the addition of DPN increased the inhibition by 63%, (i.e. from 19 to 31%), the presence of cyanide in both cases (i.e. in the presence and absence of DPN) reduced the inhibition to a low level. Therefore, the fact that cyanide effectively relieved the inhibition caused by fumarate indicates that the formation of oxalacetate was a prerequisite to the inhibition of acetylation.

The question arises as to the nature of the hydrogen acceptor involved in the oxidation of malate. Stern and Ochoa (99) have shown that malate was as effective as oxalacetate for citrate synthesis by pigeon liver extracts. The addition of pyruvate in the presence of malate greatly increased the formation of citrate, although some citrate was formed in the absence of pyruvate. It was concluded from these results that pyruvate acts as the hydrogen acceptor for malate oxidation in conformity with the following equations:

$$\text{l-malate} + DPN_{ox} \longrightarrow \text{oxalacetate} + DPN_{red} \quad \text{(malic dehydrogenase)} \quad (12)$$

$$\text{Pyruvate} + DPN_{red} \longrightarrow \text{lactate} + DPN_{ox} \quad \text{(lactic dehydrogenase)} \quad (13)$$

$$\text{Sum: l-Malate + pyruvate} \xrightarrow{DPN} \text{oxalacetate + lactate} \quad (14)$$

If pyruvate is the only hydrogen acceptor operating under the given conditions, it must be assumed that, in the absence of added pyruvate, either there is sufficient pyruvate

present in the medium to serve as a hydrogen acceptor for reduced DPN or that pyruvate is generated by the breakdown of a portion of the oxalacetate formed, since a number of experiments (Tables XIV, XV, and XVI) indicated that a high degree of inhibition of acetylation is obtained without the addition of pyruvate. Previously, under the section "Pyruvate Dismutation" page 78, the effect of fumarate in inhibiting the acetylation of sulfanilamide in the presence of acetate and ATP was compared with the effect on the acetylation associated with pyruvate dismutation (Table XII). It was found that the inhibition of acetylation was approximately the same in both cases, i.e. in the presence or absence of pyruvate. Therefore, on the basis of these results, the possibility of an alternative hydrogen acceptor for the oxidation of $DPNH_2$ cannot be excluded.

It was reported by Soodak and Lipmann (65) that oxalacetate markedly depressed the acetylation of sulfanilamide by pigeon liver extracts. Stern and Ochoa (63) reported the formation of citrate from acetate and oxalacetate by pigeon liver extracts in the presence of ATP, CoA, and Mg^{++}. While the present work was in progress Stern and Ochoa (99) reported that malate was just as effective as oxalacetate for citrate synthesis by pigeon liver extracts. It is apparent that fumarate, malate, and oxalacetate depress the formation of acetylsulfanilamide by forming citrate, thus competing for acetyl-CoA.

Since fumarate, malate, and oxalacetate inhibit the

acetylation of choline by beef brain extracts in the same manner as they inhibit the acetylation of sulfanilamide by pigeon liver extracts it must be concluded that the same mechanism is involved in both cases.

Summary

1. Fumarate, malate, and oxalacetate inhibit the synthesis of acetylcholine by beef brain extracts and the acetylation of sulfanilamide by pigeon liver extracts. Maleate has no effect on acetylcholine formation.
2. Fumarate and malate inhibit the acetylation of choline and sulfanilamide only to the extent to which they can be oxidized to oxalacetate, which competes with choline and sulfanilamide for acetyl-CoA.
3. Fumarate does not inhibit the acetylation of sulfanilamide by pigeon liver extracts in the presence of 0.1M neutral NaCN. The effect of NaCN in overcoming the fumarate inhibition is due to the formation of the cyanhydrin of oxalacetate, thus trapping the oxalacetate formed from fumarate.
4. Cyanide at a concentration of 0.1M does not inhibit the acetylation of sulfanilamide.
5. The stimulating effect of semicarbazide on acetylcholine synthesis by beef brain extracts has been confirmed.
6. 3.5×10^{-4}M Bayer-205 (suramin) completely inhibits the acetylation of sulfanilamide by pigeon liver extracts.

THE EFFECTS OF NARCOTICS ON BIOLOGICAL ACETYLATIONS

Biological acetylations, an essential activity of every living cell, has been shown to be dependent upon the available supply of high-energy phosphate (16,41). It was of interest to determine to what extent narcotics could interfere with acetylation by limiting production of the ATP required for this cellular function. The view that narcotics act by inhibiting the synthesis of ATP through the depression of oxidative metabolism would predict an inhibition of acetylation in those systems dependent upon ATP production *in situ*.

There is the possibility that narcotics may not affect the synthesis of ATP but may have a direct effect on the utilization of this compound for metabolic purposes. McElroy (137) has suggested that narcotics may act by a differential reversible denaturation of a variety of enzymes or structural proteins rather than by a specific effect on a particular enzyme. The possibility then arises that narcotics may inactivate the enzyme system responsible for acetylations, in which case no amount of ATP could possibly increase acetylation in the presence of a high narcotic concentration.

It was decided, as a preliminary measure, to ascertain what effects narcotics might have on acetylations by cell free extracts. This was done both with actively glycolyzing extracts which are capable of ATP synthesis (39), and non-glycolizing extracts to which ATP must be supplied in order to obtain acetylation.

Effect of Narconumal on Ach synthesis by cell-free extracts

Some results of typical experiments are shown in Table XVIII. It can be seen that Narconumal (5-allyl-5-isopropyl-N-methyl barbituric acid) at high concentration had no effect on the formation of acetylcholine (Ach)by a glycolyzing extract of beef brain (Exp. 1). The concentration of drug used was one and one-half times as high as that concentration which inhibited the respiration of a rat brain homogenate by 76% (Table XXVII). The fact that Ach formation was not inhibited indicated that the narcotic did not interfere with the utilization of ATP, and also that the choline acetylating enzyme system was not inactivated by the narcotic. This is also apparent in Exp. 2 (Table XVIII) where citrate is used as the acetyl donor and the only high-energy phosphate available to the system is the ATP initially added.

Harpur and Quastel (39) have shown that glucose does not inhibit Ach synthesis by brain extracts as long as conditions are present which allow glycolysis to take place. Under these conditions, the ATP used in phosphorylating glucose is more than returned to the system. However, any interference with the phosphorylation mechanism associated with glycolysis would obviously be reflected in a reduced Ach synthesis. Therefore, the fact that no inhibition of Ach synthesis was found to occur indicated that the generation of ATP in the course of anaerobic glycolysis was unaffected by the narcotic at the relatively high concentration used. Previously it was shown

TABLE XVIII

EFFECT OF NARCOTICS ON THE ACETYLATION OF CHOLINE BY BEEF BRAIN EXTRACTS at 27°C

Exp.	Additions	CO_2 output µM	Ach synthesis µg/g. powder
1	*Nil	0.2	28
	Narconumal 0.006M	0.3	28
	Glucose 0.03M	43.5	134
	Glucose 0.03M + Narconumal 0.006M	43.5	134
2**	ATP 0.002M	--	35
	Citrate 0.02M + ATP 0.002M		150
	Narconumal 0.006M + ATP 0.002M		35
	Citrate 0.02M + ATP 0.002M + Narconumal 0.006M		150

Vessel contents: 1 ml. of beef brain extract (≡ 60 mg. powder) prepared as described in the text.
$NaHCO_3$ 0.047M; sodium phosphate buffer, pH 7.5, 0.01M; choline chloride 0.0008M; eserine sulphate 0.003M.
*Exp. 1 also contained DPN, 5×10^{-4}M and ATP 0.001M.
**Exp. 2 contained NaF, 0.03M.
Gassed for 10 minutes with 93% N_2 and 7% CO_2.
Time: 1 hour plus 15 minutes equilibration.

(Table X, page 73) that Nembutal had no inhibitory effect on the acetylation of sulfanilamide by the pyruvate dismutation system present in pigeon liver extracts. It is also evident from a comparison of the values for CO_2 output shown in Table XVIII, Exp. 1, that anaerobic glycolysis was not impaired by the high narcotic concentration employed. The absence of narcotic inhibition of Ach synthesis by cell-free extracts of beef brain described above are contrary to the

findings of McLennan and Elliott (207) to the effect that Pentobarbital inhibits Ach synthesis by cell-free extracts of beef brain, but under conditions which were somewhat different from those described above.

Effect of Narcotics on Sulfanilamide acetylation by pigeon liver extracts

Table XIX shows the typical results of experiments on the effect of chloretone on the anaerobic acetylation of sulfanilamide. Although Exp. 1 and 2 (Table XIX) were carried out in the presence of air, the system was essentially anaerobic, since acetone powder extracts do not respire to an appreciable extent. It was found that acetylation proceeded just as efficiently in the presence of air as under strictly anaerobic donditions, despite the fact that no cysteine was used in preparing the extracts. In Exp. 1 and 2, 90-95% of the sulfanilamide initially added had been acetylated at the end of 90 minutes.

Chloretone (0.004M) which inhibits both respiration and acetylation in an aerobic system to the extent of 60-70% shows no effect on acetylation by an extract under anaerobic conditions, whether the substrate be pyruvate or acetate (Exp. 1 and 2, Table XIX). Exp. 3, carried out in an atmosphere of nitrogen/CO_2 indicated a small effect by nembutal but this was within experimental error. Table XX summarizes a number of experiments performed under a variety of conditions. From a consideration of results shown in this table the

TABLE XIX

EFFECT OF CHLORETONE ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT at 37°C

Exp.	Additions	Sulfanilamide added (ug)	Sulfanilamide acetylated (ug)
1	Nil	101	13
	Pyruvate 0.02M	101	45
	Pyruvate 0.02M + chloretone 0.004M	101	41
	ATP 0.001M	101	54
	Pyruvate 0.02M + ATP 0.001M	101	90
	Pyruvate 0.02M + ATP 0.001M + chloretone 0.004M	101	92
2	Acetate 0.02M	101	21
	Acetate 0.02M + chloretone 0.004M	101	21
	Acetate 0.02M + ATP 0.002M	101	93
	Acetate 0.02M + ATP 0.002M + chloretone 0.004M	101	95
3*	Acetate 0.02M + ATP 0.003M	200	155
	Acetate 0.02M + ATP 0.003M + nembutal 0.003M	200	146

Media: 1 ml. of pigeon liver extract prepared as described above. Sodium phosphate buffer, pH 7.5, 0.02M. $MgCl_2$ 0.0067M.
*Exp. 3 contained, in addition, $NaHCO_3$, 0.028M.
Gassed with 93% N_2 and 7% CO_2. Total volume 3 ml.
Experiment 1 and 2 were carried out in air.
Experimental time: 90 minutes.

Conclusion seems justified that the narcotics employed had no significant effect on the enzymes involved in acetylation, nor on their ability to utilize ATP for acetylation purposes. Secondly, the pyruvate dismutation system is apparently unaffected by high narcotic concentration as may be seen from

TABLE XX

EFFECT OF NARCOTICS ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT

Exp.	Gas phase	Substrate	Narcotic (M per litre)	ATP conc.	DPN conc.	Sulfanilamide initially added (ug)	Sulfanilamide acetylated	
							without Narcotic	With Narcotic
1	Air	Pyruvate 0.02M	Nembutal 9.6×10^{-4}M	--		202	76	76
2	N_2/CO_2	Pyruvate 0.02M	Chloretone 4×10^{-3}M	--	5×10^{-4}M	211	121	108
3	Air	Pyruvate 0.02M	Chloretone 4×10^{-3}M	0.003M	5×10^{-4}M	213	137	137
4	Air	Acetate 0.02M	Chloretone 4×10^{-3}M	0.003M	--	101	93	95
5	N_2/CO_2	Acetate 0.02M	Chloretone 5×10^{-3}M	0.0045M	--	211	193	195
6	N_2/CO_2	Acetate 0.02M	Nembutal 1.2×10^{-3}M	0.003M	--	200	155	146
7	Air	Acetate 0.02M	Pentothel 8×10^{-4}M	0.003M	--	202	139	130
		Acetate 0.02M	Amytal 8×10^{-4}M	0.003M	--	202	139	132
8	N_2/CO_2	Acetate 0.02M	Hyoscine 5×10^{-3}M	0.003M	--	202	153	148

Pigeon liver powder extracted with 0.15M KF, 0.02M $MgCl_2$.
Media: 1 ml. of liver extract (= 60 mg. powder)
Sodium phosphate buffer, pH 7.5, 0.02M.
When the gas phase was N_2/CO_2, $NaHCO_3$ 0.028M added, and vessels gassed for 10 minutes with 93% N_2 and 7% CO_2.
Total Volume: 3 ml.
Temperature: 37°C.
Experimental time: 90 minutes.

Exp. 1, 2, and 3 in Table XX. Exp. 2, Table XX shows an inhibition of 10%, which was the only instance of any inhibition observed and is bordering on the limit of experimental error.

Aerobic Acetylation of Choline

Since it had been established without doubt that narcotics have no effect on the choline acetylasee system, it was decided to investigate the effects of narcotics on choline acetylation taking place in a respiring system under aerobic conditions.

Methods

Rat brain (cerebral hemispheres) was minced with a small Latapie mincer and suspended in a solution of 0.066M nicotinamide, 0.15M KCl, and 0.03M $MgCl_2$, to make a 50% suspension. 1 ml. of suspension was added to the manometric flask. (The use of comparatively large amounts of tissue for Ach synthesis was thought advisable to show up differences in synthesis more clearly).

All manometer flasks contained the following substances (final concentrations) in a total volume of 3 ml.:

Sodium phosphate buffer, pH 7.4	0.02M
NaF	0.03M
Choline Chloride	0.001M
Eserine sulphate	0.0004M

0.2 ml. of 20% KOH was placed in the centre-well, with a roll of filter paper to absorb CO_2. Other additions were as shown in the table. Measurements of oxygen uptake were begun after a

thermal equilibration period of 5 minutes.

Acetylcholine Assay

The contents of each vessel was decanted into centrifuge tubes, and 2 ml. of 0.3M NaH_2PO_4 added to lower the pH to approximately 5. The tubes were heated for 10 minutes in boiling water, cooled, and $BaCl_2$ added to precipitate purines. Saturated $Ba(OH)_2$ solution was added to bring the pH to 7.0. After centrifugation, the supernatant was decanted and excess Na_2SO_4 added to precipitate Ba^{++}. The supernatant obtained after centrifuging was assayed by means of the leech. The above procedure, worked out by Harpur and Quastel, (39), was found to remove substances other than Ach which were active in sensitizing the leech muscle (see "Experimental Methods and Procedures" for details of Ach determination).

Choice of Substrate

The object of this work was to show that narcotics depress certain functional activities of the cell, such as acetylation, by suppressing the generation of high-energy phosphate. In mammalian tissues, the generation of high-energy phosphate is associated mainly with aerobic oxidation, glycolysis accounting for a very small part of the total. Therefore, pyruvate was chosen as the main substrate, since it would eliminate the complications of glycolysis to a large extent, and furthermore would facilitate the use of fluoride in sufficiently high concentration to inhibit ATP-ase activity

i.e. it would prevent the great losses in high-energy phosphate. Pyruvate was also used in the greater part of the work on the acetylation of sulfanilamide.

Effects of Narcotics on the Aerobic Acetylation of Choline

Typical results of some experiments dealing with the effects of narcotics on the respiration and acetylation of choline are shown in Table XXI. The addition of pyruvate (Exp. 1) increased the uptake of oxygen by 109%, which was accompanied by a 70% increase in Ach synthesis, while the addition of narconumal inhibited oxidation and acetylation in the presence of pyruvate by an approximately proportionate amount. In all cases the addition of ATP increased the acetylation of choline, both in the presence and absence of the narcotic to approximately the same extent. For example, the results from Table XXI, Exp. 2 show the formation of 21 ug. of Ach per gram tissue in the presence of pyruvate; the addition of ATP increased the formation of Ach to 30 ug. per gram tissue, to give a net gain of 9 ug. In the presence of chloretone, the addition of ATP produced a net gain of 10 ug. per gram of tissue. Consequently, it can be said that chloretone did not impede the utilization of the added ATP, since the net gain in Ach formation was the same both in the presence and absence of chloretone. Furthermore, it follows that chloretone did not inhibit the choline acetylating system. Therefore, the inhibition of acetylation by chloretone, in the absence of added ATP must have been due to the suppression of

TABLE XXI

THE EFFECTS OF NARCOTICS ON RESPIRATION AND ACETYLATION OF CHOLINE BY RAT BRAIN MINCE AT 37°C

Exp.	Pyruvate concentration	Narcotic	ATP concentration	O_2 uptake µl.	Per cent inhibition of respiration by narcotic	Choline acetylated ug./g. tissue	Per cent inhibition of acetylation by narcotic
1	Nil	---	---	420	---	10	---
	0.01M	---	---	866	---	17	---
	0.01M	Narconumal*	---	498	43	11	35
	0.01M	---	0.002M	745	---	20	---
	0.01M	Narconumal	0.002M	539	28	18	10
2	0.01M	---	---	1220	---	21	---
	0.01M	Chloretone	---	556	54	14	33
	0.01M	---	0.002M	1440	---	30	---
	0.01M	Chloretone	0.002M	792	45	24	20
3	0.03M	Chloretone	---	928	---	12	---
	0.03M	Chloretone	---	170	81	5	59
	0.03M	---	0.002M	1028	---	18	---
	0.03M	Chloretone	0.002M	307	70	10	44

Experimental Conditions: as described in the text.
*Narconumal concentration: 0.004M.
Chloretone " : 0.005M.
Time: 2 hours
Gas Phase: Air

ATP formation. The latter in all likelihood is connected with the marked inhibition of respiration caused by the narcotic, as can be seen from the parallelism between the percentage inhibitions of respiration and acetylation shown in Table XXI.

Aerobic Acetylation of Sulfanilamide by a Mixture of Rat Brain Homogenate and Pigeon Liver Extract

In order to study the effects of narcotics on brain metabolism and at the same time make use of the sulfanilamide acetylating system present in pigeon liver, it was decided to combine the two systems. Rat brain homogenate, which is incapable of acetylating aromatic amines, serves as the respiring component while pigeon liver extract contributes the acetylating system.

The initial experiments, in which both glucose and pyruvate served as substrates, gave a good rate of oxygen uptake but sulfanilamide acetylations in the neighbourhood of only 10 ug. per hour. The addition of fluoride to the homogenizing and extracting media increased the synthesis, but still not to an appreciable degree. By adjusting the ratio of brain homogenate and pigeon liver, however, a rate of acetylation was obtained which was somewhat greater and allowed a study of the acetylation process to be made.

A 25% rat brain homogenate was prepared by homogenizing the brain in a solution of the following composition: $MgCl_2$ 0.03M, KCl 0.03M, NMA 0.02M, and NaF 0.05M. The pigeon

liver powder was extracted with the same solution. 0.8 ml. of homogenate, equivalent to 200 ug. of brain, and 0.5 ml. of liver extract, equivalent to 60 mg. of powder, were added to the manometer flasks. In addition the flasks contained 0.03M sodium phosphate buffer, pH 7.5, and 105 ug. of sulfanilamide. Table XXII shows the results obtained from this experiment.

TABLE XXII

EFFECT OF THE ADDITION OF RAT BRAIN HOMOGENATE (RBH) ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT (PL)

Additions	O_2 uptake (µl.)	Sulfanilamide acetylated (µg.)
(RBH)	212	0
(PL) + acetate 0.02M	40	8
PL + acetate 0.02M + ATP 0.003M	52	75
PL + RBH + acetate 0.02M	463	43
PL + RBH + acetate 0.02M + ATP 0.003M	556	41
2 PL + RBH + acetate 0.02M + ATP 0.003M	543	50

Experimental time: 90 minutes.

This experiment indicated that the pigeon liver extract contained very little residual ATP and that the addition of rat brain homogenate to pigeon liver and acetate

increased the acetylation considerably. However in the presence of ATP and acetate, RBH caused a marked decrease in the total amount of acetylated sulfanilamide (from 75 ug. down to 41), which may have been the result of competition for "active acetate" by the formation of citrate (63) and/or aceto-acetate (65). On the other hand, the added ATP could possibly have suffered destruction by the ATP-ase present in the rat brain homogenate despite the steps taken to reduce ATP-ase activity. It was later discovered that nicotinamide which was present in fairly high concentration is itself inhibitory to sulfanilamide acetylation. Thus, at least part of the inhibition caused by the rat brain homogenate was probably due to the nicotinamide present, and therefore, in experiments subsequent to this discovery nicotinamide was present in much lower concentration.

Absence of Acetylsulfanilamide De-Acetylase in Rat Brain

There was a possibility that rat brain contained an acetylsulfanilamide "de-acetylase" which would be inimical to a high rate of acetylation of sulfanilamide. Krebs et al (196) have shown that acetylsulphomezathine is de-acetylated by pigeon liver, and also that an equilibrium exists whereby only 55% of the sulphomezathine added to pigeon liver mince, slices, or homogenates can be acetylated. However, Lipmann (160) has shown that acetylsulfanilamide is not hydrolyzed by pigeon liver preparations. The high rates of acetylation by pigeon liver extract obtained in the course of the present

work substantiated Lipmann's findings with regard to extracts.

To determine whether or not rat brain homogenate was capable of the hydrolysis of acetylsulfanilamide the following experiment was carried out:

Rat brain homogenate was prepared in the same manner as that which was used in conjunction with pigeon liver extract to obtain the results shown in Table XXII. A saturated water solution of acetylsulfanilamide* was prepared of which 0.5 ml. was added with 0.8 ml. of the rat brain homogenate to the manometer flasks in a total volume of 3 ml. The flasks also contained sodium phosphate buffer, pH 7.5, 0.02M. Incubation was carried out at 37°C in the presence and absence of pyruvate, with and without ATP. After 90 minutes the flask contents were analyzed for the appearance of sulfanilamide. In every case there was no trace of sulfanilamide as indicated by sulfanilamide determination, thus showing that the acetylsulfanilamide had not been hydrolyzed by the brain tissue.

The Effect of Chloretone on Respiration and Acetylation of Sulfanilamide

Table XXIII shows the results obtained upon the addition of chloretone to the brain-liver system previously described (with the exception that nicotinamide was omitted from the medium). It may be seen that chloretone at high concentration depressed respiration almost completely.

The addition of acetate to the mixture of rat brain

*Kindly supplied by Dr. S. Baker, Montreal General Hospital, Research Institute.

TABLE XXIII

EFFECT OF CHLORETONE ON RESPIRATION AND ACETYLATION OF SULFANILAMIDE BY A MIXTURE OF RAT BRAIN HOMOGENATE (RBH) AND PIGEON LIVER EXTRACT (PL) AT 37°C

Additions	O_2 uptake in ul.	Sulfanilamide acetylated ug.
RBH + PL	122	21
RBH + PL + chloretone 0.004M	20	15
RBH + PL + acetate 0.02M	222	44
RBH + PL + acetate 0.02M + chloretone 0.004M	11	21
RBH + PL + acetate 0.02M + ATP 0.002M	220	46
RBH + PL + acetate 0.02M + ATP 0.002M + chloretone 0.004M	71	38

Experimental conditions: As described in the text.
Experimental Time: 90 minutes.

homogenate and pigeon liver extract increased the acetylation of sulfanilamide by more than 100%. However, when chloretone was present the addition of acetate failed to increase the acetylation of sulfanilamide. It will be seen that chloretone at a concentration of 0.004M, almost completely inhibited respiration, from which it can be concluded that no appreciable formation of ATP took place when chloretone was present. Hence it is understandable that the addition of acetate under these conditions was unable to give rise to an increased formation of acetylsulfanilamide, in view of the deficiency of ATP. Of

greater interest was the fact that the addition of ATP almost completely overcame the inhibition of acetylation by chloretone in the presence of acetate while at the same time the inhibition of respiration was relieved to only a slight extent.

Thus it is apparent that a narcotic cannot inhibit acetylation to an appreciable degree when the acetylation process is rendered independent of respiration for its requirement of high-energy phosphate by the addition of ATP to the system, and provided that the supply of acetyl groups is adequate for acetylation purposes. In practice, however, allowances must be made for such factors as ATP-ase activity which will, over a period of time, diminish the concentration of ATP and so may not make possible the complete reversal of the narcotic inhibition of acetylation.

Comparison of Pyruvate and Acetate as Acetyl Precursors for Sulfanilamide Acetylation; Effects of Chloretone

An experiment was performed for the purposes of comparing the efficiency of pyruvate with that of acetate as acetyl precursor for sulfanilamide acetylation by the mixture of rat brain homogenate and pigeon liver extract previously described. Hitherto, a mixture of the two tissue preparations had been used in which the relative proportions were 200 mg. of rat brain and pigeon liver extract equivalent to 60 mg. of powder. By reducing the quantity of rat brain to 100 mg. it was found that a much higher rate of sulfanilamide acetylation was obtained. It is probable that with the former ratio of rat

brain to pigeon liver the formation of citrate was taking place at the expense of acetylsulfanilamide.

The results of this experiment are tabulated below (Table XXIV). It may be seen that the addition of acetate to the mixture of rat brain and pigeon liver increased the acetylation of sulfanilamide by 125%, while at the same time not increasing the uptake of oxygen.

TABLE XXIV

EFFECT OF CHLORETONE ON RESPIRATION AND ACETYLATION OF SULFANILAMIDE BY A MIXTURE OF RBH AND PL AT 37°C

(Time: 2 hours)

Substrate	Chloretone	O_2 uptake µl.	Per cent inhibition of respiration	Sulfa* acetylated ug.	Per cent inhibition of acetylation
Nil	-	183	-	36	-
Nil	0.004M	52	71	27	25
Acetate 0.02M	-	185	-	81	-
Acetate 0.02M	0.004M	36	81	27	66
Pyruvate 0.02M	-	345	-	108	-
Pyruvate 0.02M	0.004M	37	90	27	75

Media: RBH and PL prepared as described on page 108.
RBH added: equivalent to 100 mg. of brain (wet weight).
PL extract: equivalent to 60 mg. powder.
Sodium phosphate buffer, pH 7.5, 0.02M.
*Sulfanilamide added: 414 ug.
Total volume 3 ml.

It is possible that the oxidation of endogenous substrates generated the high-energy phosphate required for acetylation, and that the added acetate served only as a source of acetyl groups.

The addition of pyruvate, on the other hand, increased the acetylation by 200%, and also almost doubled the uptake of oxygen. Thus pyruvate oxidation provided both high energy phosphate and acetyl groups for acetylation purposes.

The effect of chloretone on respiration and acetylation in the absence of added substrate was similar to that observed previously (Table XXIII). Although respiration was inhibited by 71% (Table XXIV) acetylation was inhibited by only 25%. It may be of some significance that the addition of chloretone reduced the acetylation in each case to a common level, i.e. 27 ug. in 2 hours. The probability exists that this common level represents the acetylation which arises from residual ATP present in the system, and which is unaffected by the chloretone.

It was observed that chloretone and other narcotics inhibited the respiration and acetylation to a much greater extent when pyruvate was being oxidized than was the case when acetate was added as substrate. This of course was due to the fact that the rates of oxidation and acetylation were higher in the presence of pyruvate, and that the high narcotic concentration employed reduced both processes to low basic levels.

This can be seen from Table XXIV which indicates that acetylation was depressed by chloretone to 27 ug./2 hours in the presence of both acetate and pyruvate, and the uptake of oxygen was reduced to 36 ul./2 hours and 37 ul./2 hours for acetate and pyruvate, respectively.

It was also found that the addition of ATP was effective in overcoming the narcotic inhibition of acetylation to a large extent when acetate was present but that ATP had little effect in the presence of pyruvate.

As a consequence of these observations the work could be conveniently sub-divided on the basis of added substrate. Therefore, the following section deals with the effects of narcotics on respiration and sulfanilamide acetylation in the presence of acetate as substrate.

Effects of Narcotics on Respiration and Acetylation in the Presence of Acetate

A 25% homogenate of rat brain was prepared by homogenizing the cerebral hemispheres in a solution composed of 0.06M KCl, 0.04M NAA, and 0.04M $MgCl_2$. 0.5 ml. of homogenate was added to the manometer flask, ie. 125 mg. of brain per flask. Pigeon liver acetone powder was extracted by rubbing it up in 0.15M KF. After centrifugation at 19,000 g for 5 minutes, the supernatant was filtered through glass wool, and 0.8 ml. of extract, equivalent to 60 mg. of powder, was pipetted directly into the manometer flask. The flasks also contained 200 ug. of sulfanilamide (unless otherwise stated), sodium phosphate buffer,

pH 7.5, 0.02M, and other additions as indicated in the tables. Acetate was added as the sodium salt.

The final fluoride concentration was 0.04M. In the present work it has been found that a fairly high fluoride concentration is essential to prevent breakdown of labile phosphates, otherwise a very low acetylation is obtained. For example, on one occasion the pigeon liver was extracted with 0.15M KCl in place of 0.15M KF. With fluoride absent 247 ul. of O_2 were taken up by the mixture of rat brain and pigeon liver extract in the presence of pyruvate, and 18 ug. of sulfanilamide acetylated, whereas the addition of fluoride to the vessel to a final concentration of 0.04M resulted in 348 µl. of O_2 being taken up and 81 µg. of sulfanilamide acetylated. Hence, under these conditions the fluoride accounted for almost a five-fold increase in acetylation and also increased the O_2 uptake.

Acetate as Substrate

It may be seen from Tables VIII, and XXII that pigeon liver acetylated very little sulfanilamide in the presence of acetate unless ATP was supplied. However, when a system which is capable of generating high energy phosphate was added, such as rat brain homogenate a large increase in acetylation was secured; it is apparent that the rat brain homogenate provided the ATP necessary for acetylation to take place since a similar effect was obtained by replacing rat brain homogenate with ATP. Similarily from Table XXIV it may be seen that acetate gave

rise to acetylated sulfanilamide in the absence of ATP provided rat brain homogenate was present.

It would be expected that when an inhibition of oxidation by a narcotic results in a decreased acetylation due to insufficient ATP formation, the addition of ATP to the system should meet the requirements for acetylation and hence reverse the inhibition of acetylation caused by the narcotic. However, a tissue preparation which is actively respiring can, to some extent, withstand the loss of ATP resulting from ATP-ase activity, particularly when additional ATP is supplied. When a narcotic is present in high concentration, there will be no appreciable formation of ATP to replace the losses due to ATP-ase activity. Therefore, a complete reversal of narcotic effect on acetylation may not be attainable since ATP will be continuously removed through ATP-ase activity, even in the presence of fluoride.

Table XXV shows the effect of the addition of a high concentration of chloretone to a system which was actively acetylating sulfanilamide. Commensurate with the marked inhibition of respiration there was a drop in acetylation. It will be observed, however, that in the presence of added ATP, the extent of the inhibition by chloretone was greatly diminished. From Table XXV, Exp. 2 it may be seen that chloretone inhibited respiration by 60% and acetylation by 70%. In the presence of 0.003M ATP chloretone produced the same inhibition of respiration, but the inhibition of acetylation was reduced

TABLE XXV

EFFECTS OF CHLORETONE ON RESPIRATION AND ACETYLATION OF SULFANILAMIDE BY A MIXTURE OF PIGEON LIVER EXTRACT AND RAT BRAIN HOMOGENATE IN THE PRESENCE OF SODIUM ACETATE (0.02M) at 37°C

Exp.	Additions: Chloretone	Additions: ATP	O_2 uptake µl.	Per cent inhibition of respiration by chloretone	Sulfa* acetylated µg.	Per cent inhibition of acetylation by chloretone
1	-------	---	258	--	67	--
	0.004M	---	101	60	27	60
	-------	0.002M	286	--	90	--
	0.004M	0.002M	164	43	59	34
2**	-------	--½	356	--	87	--
	0.004M	---	143	60	27	70
	-------	0.003M	345	--	121	--
	0.004M	0.003M	158	60	87	20

Experimental conditions as described on page 116.
*Sulfanilamide added: 200 ug.
**Exp. 2 contained DPN, 0.00025M.
Experimental time: 90 minutes.

from 70 to 20%. Thus it appears that the ATP added had substituted for the ATP which normally would have been produced were it not for the inhibition of oxidation exercised by the narcotic. In effect, the addition of ATP overcame the inhibition of acetylation by the narcotic.

As a corollary to the effect of ATP in overcoming the chloretone inhibition of acetylation, it follows that the

narcotic was not exercising a general enzymic inhibition, but that its effect was, in this case, specifically located in the respiratory process, the inhibition of which was not relieved by the addition of ATP (Table XXV, Exp. 2).

Pyruvate as Substrate

The addition of pyruvate alone to pigeon liver extract gives rise to a high rate of acetylation under optimal conditions. However, when rat brain homogenate was added to pigeon liver in the presence of pyruvate under anaerobic conditions pyruvate dismutation was somewhat depressed (Table XXVI). It may be seen(Exp. 3, Table XXVI) that the

TABLE XXVI

INHIBITION OF PYRUVATE DISMUTATION AND ACETYLATION OF SULFANILAMIDE BY RAT BRAIN HOMOGENATE UNDER ANAEROBIC CONDITIONS AT 37°C

Exp.	Additions	CO_2 output $\mu l.$	Sulfa acetylated µg.	Per cent inhibition of acetylation
1	P.L. + pyruvate 0.02M	399	67	--
	P.L. + pyruvate 0.02M + RBH		36	46
2	P.L. + pyruvate 0.02M	558	65	--
	P.L. + pyruvate 0.02M + RBH	179	35	46
3	P.L. + DPN*	65	13	--
	P.L. + pyruvate 0.02M	157	66	--
	P.L. + DPN + pyruvate 0.02M	186	121	--
	P.L. + DPN + pyruvate 0.02M +RBH	211	90	26

*DPN 2.5 x 10^{-4}M, $NaHCO_3$ 0.028M. Gassed with 93% N_2 and 7% CO_2. Other conditions as described on page 116. Sulfanilamide added: 200 ug.

addition of DPN overcomes the inhibition to a large extent, indicating that rat brain DPN-ase was likely responsible for the inhibition under anaerobic conditions.

Under aerobic conditions, however, the addition of rat brain homogenate secured a large increase in the acetylation of sulfanilamide which was accompanied by a high rate of O_2 uptake. The addition of a narcotic to a mixture of pigeon liver extract and rat brain homogenate respiring in the presence of pyruvate was responsible for a marked inhibition of both oxidation and acetylation (Table XXVII, Exp. 1 and 2). While chloretone had no appreciable effect on the acetylation of sulfanilamide by pigeon liver extract alone, acetylation taking place in the respiring system was inhibited by 85% (Exp. 1) and in Exp. 2 by 67%, and oxygen uptake was inhibited to a similar degree. Whereas ATP was largely effective in overcoming the narcotic inhibition of acetylation in the presence of acetate, (Table XXV), the same was not true when pyruvate was the substrate (Table XXVII).

Since narcotics appear to inhibit the pyruvic oxidase system (140,142,147), the breakdown of pyruvate to acetate and CO_2 cannot occur in the presence of a high narcotic concentration. Therefore, no amount of ATP could be effective in relieving the inhibition of acetylation due to the deficiency of 2-C fragments required for acetylation purposes. Indeed, the fact that ATP is effective in relieving the chloretone inhibition of acetylation in the presence of acetate but not in

TABLE XXVII

EFFECT OF NARCOTICS ON OXIDATION AND ACETYLATION BY A MIXTURE OF RAT BRAIN HOMOGENATE AND PIGEON LIVER EXTRACT IN THE PRESENCE OF PYRUVATE (0.02M) AT 37°C

Exp.	Additions	O_2 uptake µl.	Per cent inhibition of O_2 uptake	Sulfa acetylated µg.	PER CENT inhibition of acetylation
1	PL	38	--	63	--
	PL + chloretone 0.004M	10	--	59	6
	PL + RBH	425	--	95	--
	PL + RBH + chloretone 0.004M	103	76	14	85
	PL + RBH + ATP 0.002M	443	--	122	--
	PL + RBH + ATP 0.002M + chloretone 0.004M	153	65	32	77
2	PL + RBH	365	--	94	--
	PL + RBH + chloretone 0.004M	156	60	31	67
	PL + RBH + ATP 0.002M	550	--	125	--
	PL + RBH + ATP 0.002M + chloretone 0.004M	237	57	54	57
3	PL	38	--	72	--
	PL + RBH	454	--	167	--
	PL + RBH + Nembutal 0.005M	275	39	104	38

Experimental Conditions: See page 116.

presence of pyruvate may perhaps be taken as additional evidence that narcotics inhibit the pyruvic oxidase system, as indicated by the lack of availability of the 2-C fragments for acetylation purposes.

The Anaerobic Oxidation of Pyruvate

It has been shown by Michaelis and Quastel (140) that pyruvate in the presence of chloretone, can be oxidized anaerobically by ferricyanide when pyocyanine is also present. This finding has been confirmed in the present work.

The technique employed was that of Quastel and Wheatley (172) which is based on the fact that in the reduction of 1 mol. of ferricyanide 1 mol. of acid is formed, giving rise in bicarbonate media to 1 mol. of CO_2 which is estimated manometrically. The relevant equations are:

$$H + Fe(CN)_6^{---} \longrightarrow H^+ + Fe(CN)_6^{----}$$

$$H^+ + HCO_3^- \longrightarrow CO_2 + H_2O$$

The addition of alkaline ferricyanide solution to rat brain homogenate in bicarbonate-pyruvate medium in an atmosphere of N_2/CO_2 results in very little evolution of CO_2. The addition of pyocyanine to the medium followed by ferricyanide, results in a large increase in CO_2 output. Pyocyanine is reduced in the presence of brain tissue by pyruvate and the reduced pyocyanine is oxidized directly by ferricyanide. Details of the technique may be obtained from the original papers. The results of one experiment, shown in Table XXVIII, indicate that pyruvic dehydrogenase is not affected by chloretone.

It was intended to determine to what extent the anaerobic oxidation of pyruvate by a mixture of brain and liver

TABLE XXVIII

ANAEROBIC OXIDATION OF PYRUVATE BY BRAIN HOMOGENATE IN THE PRESENCE OF FERRICYANIDE AND PYOCYANINE (at 37°C)

Additions	CO_2 output ul.
Ferricyanide	26
Pyruvate + Ferricyanide	63
Pyocyanine + Ferricyanide	80
Pyocyanine + ferricyanide + chloretone	87
Pyruvate + pyocyanine + ferricyanide	252
Pyruvate + pyocyanine + ferricyanide + chloretone	244

Vessel contents: Rat brain (1.5 gm) homogenized in saline, washed once with saline and suspended in 10 ml. of 0.16M NaCl; 1 ml. of suspension added to each manometer flask. $NaHCO_3$, 0.028M; Pyocyanine 3.2 mg.%; ferricyanide, 0.005% in 0.028M $NaHCO_3$; pyruvate, 0.02M. (All concentrations are final). Chloretone 0.004M. Total Volume: 3 ml. Gassed for 10 minutes with 93% N_2 and 7% CO_2. (Ferricyanide was tipped in from the side arm after a 15 minute equilibration period, during which negligible CO_2 was evolved. The manometers were adjusted to zero before tipping in the ferricyanide).
Time: 60 minutes.

extract could give rise to acetylation, which would be an indication of the high energy phosphate generated, if any, by the initial dehydrogenation of pyruvate. However, it was found that ferricyanide was highly inhibitory to the acetylation process (Table XXIX) and therefore could not be employed in the above manner.

TABLE XXIX

THE INHIBITION OF ACETYLATION BY FERRICYANIDE (at 37°C)

Additions	Sulfanilamide acetylated ug.	Per cent inhibition of acetylation by ferricyanide
Acetate 0.02M + ATP 0.003M	171	
Acetate 0.02M + ATP 0.003M + $K_3Fe(CN)_6$ 0.01M	43	75
Pyruvate 0.02M + ATP 0.00075M	81	
Pyruvate 0.02M + ATP 0.00075M + $K_3Fe(CN)_6$ 0.01M	16	80

Vessel contents: 1 ml. of pigeon liver extract (60 mg. powder). $NaHCO_3$, 0.028M; sodium phosphate buffer, pH 7.5.
Total Volume: 3 ml.
Gassed with 93% N_2 and 7% CO_2.
Time: 60 minutes.

Ferricyanide has been shown to have little effect on anaerobic glycolysis (173) and on the respiration of tumor tissue (174) at a concentration of 0.01M; therefore it was rather surprising to find that ferricyanide inhibited the acetylation reaction. Possibly ferricyanide may act by oxidizing the -SH group of Coenzyme A. If so, ferricyanide should also inhibit the formation of citrate under similar conditions. The compound has in fact, been used by Anson (186) for the determination of -SH groups in proteins and by Mason (187) for the determination of glutathione.

Rat-Brain-Pigeon Liver Preparation and the Tricarboxylic Acid Cycle

An accumulation of citrate and α-ketoglutarate was found by Coxon, et al (175) to accompany the oxidation of pyruvate by dialysed, finely ground homogenates of pigeon brain in a medium containing fumarate, Mg^{++}, and ATP. When fumarate was absent an accumulation of acetate was observed. These authors also reported that the brain homogenate did not oxidize citrate but could oxidize cis-aconitate to a small extent. Indeed, one of the main arguments, in the past, against the idea that a tricarboxylic acid cycle was involved in the oxidation of pyruvate by brain tissue was the fact that the increased oxygen uptake observed on addition of fumarate as well as pyruvate was not observed with citrate, which therefore could not be an intermediate.

During the present work it was found that no increase in oxygen uptake occurred when citrate was added to rat brain homogenate, which confirms the observations of Coxon, et al (175) and others. However, when an extract of acetone-dried pigeon liver was added to the brain homogenate the addition of citrate increased the uptake of oxygen by more than 50%. Apparently the addition of pigeon liver extract provided some factor, (or factors) which enabled the oxidation of citrate to take place. The nature of the factor involved was not investigated further, and mention is made of it here only for the reason that it provides additional information as regards

the efficiency of the respiratory process in the brain-liver preparation. Furthermore, the effects of malonate in the presence of various substrates (Table XXX) indicate that the tricarboxylic acid cycle is functioning completely in this system (See also Table XXXI).

The Effect of Fumarate and Malonate on the Aerobic Acetylation of Sulfanilamide

Methods

Rat brain (cerebral hemispheres) was homogenized in KCl 0.06M, $MgCl_2$ 0.04M, and NAA 0.017M to make a 25% homogenate; 0.5 ml. (125 mg. of brain) was added to the manometer flasks. Acetone-dried powder of pigeon liver was extracted by grinding it in 0.15M KF. After centrifugation at 19,000 g for 5 minutes the supernatant was filtered through glass wool, and 0.8 ml. (equivalent to 60 mg. powder) was pipetted directly into the manometer flasks. In addition, the flasks contained 200 ug. of sulfanilamide, sodium phosphate buffer (pH 7.5) 0.02M, and other materials as indicated in the tables, in a total volume of 3 ml. 0.2 ml. 20% KOH and a roll of filter paper were added to the centre well of the flasks. After a thermal equilibration period of 5 minutes the manometer stop-cocks were closed and measurement of oxygen uptake was begun. All experiments were carried out in the presence of air.

The Action of Fumarate

It was shown previously (page 88, Table XV) that under anaerobic conditions fumarate inhibited the acetylation of sulfanilamide by pigeon liver extract in the presence of

acetate and ATP as acetylating agents. This was explained on the basis of the anaerobic oxidation of fumarate to oxalacetate, which in the presence of acetyl-CoA forms citrate, thus competing with sulfanilamide for Acetyl-CoA (page 96).

The oxidation of pyruvate by the brain-liver system used in the present work has been shown to enhance the acetylation of sulfanilamide. It might be expected that the addition of fumarate to the system in the presence of pyruvate would favour the formation of citrate with the result that sulfanilamide acetylation would be somewhat reduced due to competition for Acetyl-CoA by oxalacetate and sulfanilamide.

It may be seen from Table XXX (Exp. 1 and 2) that the addition of a low concentration of fumarate did, in fact, inhibit the acetylation of sulfanilamide. At the same time fumarate increased the uptake of oxygen in the presence of pyruvate by 50% (Exp. 1 and 2) indicating that pyruvate was probably being oxidized via the tricarboxylic acid cycle. Furthermore, the addition of fumarate alone (Exp. 2) increased the respiration to a greater extent than did pyruvate alone, but at the same time gave rise to less sulfanilamide acetylated. This is consistent with the results of Coxon, et al (see page 126). The increased acetylation by fumarate could only be explained on the basis of the partial breakdown of some oxalacetate, an oxidation product of fumarate, to provide acetyl fragments for the acetylation of sulfanilamide.

TABLE XXX

THE EFFECT OF MALONATE ON THE FUMARATE INHIBITION OF THE ACETYLATION OF SULFANILAMIDE BY A MIXTURE OF RAT BRAIN HOMOGENATE AND PIGEON LIVER AT 37°C

Exp.	Additions	O_2 uptake (µl.)	Sulfanilamide acetylated (µg.)	Per cent Inhibition of Acetylation by fumarate
1	Pyruvate	439	130	--
	Pyruvate + Fumarate	666	90	31
2	Nil	316	36	--
	Pyruvate	568	121	--
	Fumarate	724	72	--
	Pyruvate + fumarate	851	94	22
	Malonate	208	45	--
	Fumarate + malonate	627	94	--
	Pyruvate + malonate	374	121	--
	Pyruvate + fumarate + malonate	785	117	3

Experimental Conditions as described on page 27.
Substrate concentrations: Pyruvate 0.02M, fumarate 0.006M, malonate 0.02M.
Duration: 90 minutes.

The action of malonate

It has been shown by Quastel and Woolridge (179) that malonate inhibits succinic dehydrogenase, thus preventing the oxidation of succinate, without affecting other enzymes concerned in the tricarboxylic acid cycle, and Elliott and Greig (208) have shown that malonate (0.02M) inhibits succinate oxidation at least 90% in tissue slices. Furthermore, one of the most important facts used in the proof of a tricarboxylic acid cycle in tissues (other than brain)

has been the demonstration of the formation of succinate from fumarate in the presence of excess malonate (213). The prediction could be made that the addition of malonate would tend to overcome the fumarate inhibition of acetylation by blocking the tricarboxylic acid cycle at the succinate stage (179), thus diverting acetyl-CoA towards the formation of acetylsulfanilamide. This, indeed, proved to be the case (Table XXX, Exp. 2).

Malonate, when added in the presence of pyruvate, decreased the uptake of oxygen appreciably but did not increase the acetylation. This was to be expected since in the absence of added oxalacetate or a precursor of oxalacetate there was probably insufficient formation of citrate from pyruvate to interfere with sulfanilamide acetylation, in which case the addition of malonate would have no effect. However, when fumarate was present the addition of malonate in every instance (Exp. 2), enhanced the acetylation of sulfanilamide, indicating that in the brain-liver preparation there was formation of citrate which was further oxidized through the tricarboxylic acid cycle. Even in the absence of added substrate, the addition of malonate increased the acetylation of sulfanilamide to a slight extent.

Coincident with the increase in acetylation caused by the addition of malonate, there was in every case a diminution of the oxygen take up by the system. When pyruvate was being oxidized the addition of a low concentration of fumarate increased the oxygen consumption by 33% and decreased the

acetylation of sulfanilamide by 22%. However, upon the addition of malonate the picture was completely reversed, i.e. the uptake of oxygen was decreased by 10% and the inhibition of acetylation was completely overcome (within experimental error).

The inhibition of sulfanilamide acetylation by fumarate was not obtained, however, with all tissue preparations (Table XXXI, Exp. 1). A possible explanation is that the enzyme concerned with sulfanilamide acetylation remained fully saturated, and that the addition of fumarate merely increased the rate of disappearance of pyruvate.

The effects of fumarate and malonate on the acetylation of sulfanilamide are similar to those observed by Lehninger (72,73), who found that the addition of fumarate to washed liver cells oxidizing octanoate diminished the synthesis of aceto-acetate, while the addition of malonate increased the synthesis of aceto-acetate by liver cells oxidizing pyruvate. These experiments illustrate the key role of Acetyl-CoA in metabolic reactions.

Behaviour of Chloretone in the Presence of Fumarate and Succinate.

It has been shown that the oxidation of succinate to fumarate is coupled with the formation of high-energy phosphase (209,210). Recently, Hersey and Ajl (211) have shown that the single step oxidation of succinate to fumarate by cell-free extracts of E. coli results in the esterification

of inorganic P^{32} into ATP. Quastel and Wheatley (131) observed that narcotics inhibit the oxidation of glucose, lactate, and pyruvate, but have no effect on the oxidation of succinate. The results obtained by Barrett (212) in reducing the depth of narcosis by succinate administration are in agreement with the above data.

It would be expected that, if the oxidation of succinate is coupled with the formation of high energy phosphate, the addition of succinate to the brain-liver preparatinn previously described should give rise in the presence of chloretone to the acetylation of sulfanilamide.

The results shown in Table XXXI (Exp. 2) indicate that succinate was just as efficient as pyruvate in stimulating sulfanilamide acetylation. Furthermore, succinate gave rise to a rapid rate of respiration which was not increased by the addition of pyruvate, although the acetylation of sulfanilamide was increased to a slight extent. The fact that the oxidation of succinate accounted for as much oxygen being taken up as was the case with succinate and pyruvate combined seems to indicate that both succinate and pyruvate were oxidized through the tricarboxylic acid cycle.

Of some significance is the fact that when succinate was present (Table XXXI, Exp. 2 and 3) the inhibition by chloretone of acetylation as well as respiration was to a large extent diminished. This is illustrated in a striking manner in Exp. 2 where the sulfanilamide acetylated in the

TABLE XXXI

THE INHIBITION OF OXIDATION AND ACETYLATION BY CHLORETONE IN THE PRESENCE OF PYRUVATE, FUMARATE, AND SUCCINATE AT 37°C

Exp.	Additions	O_2 uptake ul.	Inhibition of oxidation Per cent	Sulfanilamide acetylated (ug)	Inhibition of Acetylation Per cent
1	Nil	180	---	34	---
	Pyruvate	323	---	149	---
	Pyruvate + Fumarate	608	---	153	---
2	Pyruvate	326	---	146	---
	Pyruvate + chloretone	95	71	22	85
	Succinate	714	---	148	---
	Succinate + chloretone	274	62	58	61
	Pyruvate + succinate	729	---	162	---
	Pyruvate + succinate + chloretone	241	67	67	58
3	Nil	211	---	34	---
	Chloretone	96	54	16	53
	Fumarate	536	---	101	---
	Fumarate + chloretone	109	80	16	84
	Succinate	777	---	151	---
	Succinate + chloretone	302	61	56	63

Experimental Conditions: As detailed on page 127.
Rat brain homogenate and pigeon liver extract present throughout.
Substrate concentrations: Pyruvate 0.02M, fumarate 0.02M, succinate 0.02M, Sulfanilamide added: 202 µg.
Chloretone: 0.0047M.
Duration: 90 minutes.

presence of chloretone was only 22 ug. per 90 minutes when pyruvate was the substrate, but three times this value with succinate or pyruvate plus succinate as substrate. In Exp. 3 there was no increase in acetylation when fumarate was added

to a system already containing chloretone, nor was there any appreciable increase in oxygen uptake, i.e. the oxidation of added fumarate was almost completely blocked. Such was not the case when succinate was added, presumably because succinic dehydrogenase was still able to function. Although the inhibitions of oxidation and acetylation in this case were 61% and 63%, respectively, these inhibitions were largely due to the effect of chloretone on the oxidation of substrates other than succinate. In absolute terms, the one-step oxidation of succinate to fumarate accounted for the acetylation of 40 ug. of sulfanilamide. The implication of this fact is that in the oxidation of succinate to fumarate there must have been a generation of high-energy phosphate in the presence of a narcotic concentration which was sufficiently high to block the oxidation of fumarate and therefore the functioning of the tricarboxylic acid cycle.

This effect of succinate is rather surprising in view of the findings of Furchgott and Shore (176) and others (177,178) that succinate is oxidized, for the most part, only as far as fumarate and malate, in brain and liver separately. The above authors also show that phosphorylation in cardiac muscle slices is depressed by succinate, and conclude from their investigations that "the energy provided by the oxidation of succinate cannot be used for the maintenance of any of the important energy-requiring metabolic processes which we have studied--acetylcholine synthesis in brain, urea synthesis in

liver, and phosphorylations in cardiac and intestinal smooth muscle."

Such conclusions are difficult to reconcile with the results which have been obtained with succinate in the acetylation of sulfanilamide.

The Effect of 2,4-Dinitrophenol on the Aerobic Acetylation of Sulfanilamide in the Presence of Succinate

Previously it was shown that succinate was able to give rise, to some extent, to the acetylation of sulfanilamide in the presence of a concentration of chloretone (0.0047M) which was effective in completely blocking the oxidation of fumarate and completely inhibiting the acetylation arising therefrom. Presumably, the sulfanilamide acetylation due to succinate, which took place in the presence of chloretone, was dependent upon the generation of high-energy phosphate coupled with succinate oxidation. Should this be so it can be concluded that a high narcotic concentration does not inhibit the esterification of inorganic phosphate which is coupled with the oxidation of succinate.

2,4-Dinitrophenol has been shown to "uncouple" respiration from phosphorylation (96). Furthermore, it was shown (page 71, Table X) that 2,4-DNP, at a concentration of 0.001M, had no appreciable inhibitory effect on the acetylation of sulfanilamide by pigeon liver extract with acetate and ATP as acetylating agents, i.e. 2,4-DNP did not interfere with the utilization of ATP for acetylation purposes. In view of these

data it was decided to ascertain what effects 2,4-DNP might have on the aerobic acetylation of sulfanilamide in the presence of succinate.

The same brain-liver preparation was used in this investigation as was used to obtain the results shown in Table XXXI, with the exception that a different acetone-dried pigeon liver powder was employed. The experimental conditions are described on page 127.

TABLE XXXII

THE INHIBITION BY 2,4-DNP OF THE AEROBIC ACETYLATION OF SULFANILAMIDE IN THE PRESENCE OF SUCCINATE (at 37°C)

Additions	O_2 uptake ul.	Inhibition of respiration Per cent	Sulfanilamide acetylated ug.	Inhibition of Acetylation Per cent
Nil	204	--	44	--
2,4-DNP 10^{-4}M	140	31	9	75
Succinate 0.02M	695	--	117	--
Succinate 0.02M + 2,4-DNP 10^{-4}M	310	55	5	96
Succinate 0.02M + 2,4-DNP 2×10^{-4}M	240	65	5	96

Experimental conditions: as described on page 127.
Vessel contents: Rat brain homogenate and pigeon liver extract (see page 127). Sodium phosphate buffer, pH 7.5, 0.02M, Sulfanilamide 207 ug. Sodium succinate and 2,4-DNP (aqueous solution) as shown in the table.
Experimental time: 90 minutes.

2,4-DNP at the relatively high concentration of 10^{-4}M almost completely inhibited the acetylation of sulfanilamide in the presence of succinate (Table XXXII). This effect was undoubtedly due to the "uncoupling" of phosphorylation from oxidation rather than an interference with ATP utilization or destruction of ATP. On the basis of the contrasting effects shown by chloretone and 2,4-DNP the conclusion seems justified that the oxidation of succinate in the presence of chloretone was coupled with the formation of high-energy phosphate. Moreover, an important difference in the mechanism of action of narcotics as compared with "uncoupling" agents is implied. The comparative effects of chloretone on oxidation and acetylation in the presence of succinate and other substrates indicate that the site of narcotic action is in the respiratory process, while the uncoupling agents are known to exert their effects essentially, not on respiration, but on phosphorylation.

Relative Effects of Narcotics on Respiration and Acetylation

According to the theory that narcotics act by inhibiting the generation of high-energy phosphate through the depression of oxidative metabolism it would be expected that a relationship should obtain between the inhibition of oxidation and depression of acetylation.

To ascertain this relationship experiments were carried out with the following narcotics at varied

concentrations:

Nembutal (Pentobarbital)	5-ethyl-5-(1 methylbutyl) barbituric acid
Amytal (Amobarbital)	5-ethyl-5-isoamylbarbituric acid
Chloretone	Trichlorbutanol

Methods

Rat brain (cerebral hemispheres) was homogenized in KCl 0.06M, $MgCl_2$ 0.04M, and NAA 0.017M to give a 20% homogenate. 0.5 ml. of homogenate (100 mg. brain) was added to the manometer flasks after all other additions had been made. Acetone powder of pigeon liver was extracted by grinding with 0.15M KF in the usual manner. 0.8 ml. (equivalent to 60 mg. powder) was pipetted directly into the manometer flasks. In addition, the manometer flasks contained 200 ug. sulfanilamide, sodium pyruvate 0.02M, and narcotics as indicated in the accompanying figures, in a total volume of 3 ml. 0.2 ml. 20% KOH and a roll of filter paper were added to the centre well of the flasks for CO_2 absorption. After a 5 minute thermal equilibration period, the manometer stop-cocks were closed and measurements of oxygen uptake were begun. All experiments were carried out in the presence of air, and for a period of 90 minutes.

The sodium salts of the barbiturates employed in this work were dissolved in water and neutralized with HCl before use. The narcotic solution was added directly to the vessels, and was in contact with the tissue for approximately 10 minutes (from the time the brain homogenate was added until

thermal equilibration was complete) before measurement of O_2 uptake was begun. It may be seen from Fig. 4 that the inhibition of O_2 uptake was immediate and remained fairly steady throughout the whole period of time. The stabilization of the inhibition may in part be due to the high K^+ concentration used (0.05M in these experiments), since Jowett and Quastel (132) have shown that with a low K^+ concentration (0.002M)the inhibition of respiration by a narcotic increases with time, while at high K^+ concentration the inhibition rapidly reaches a steady state.

The Relative Effects of Narcotic Concentration on Respiration and Acetylation

In Fig. 4 to 7 are shown the results of experiments in which the narcotic concentration was varied. Fig. 4, in which oxygen uptake is plotted as a function of time, shows the results of an experiment with Nembutal at various concentrations i.e. 3,6,9,12, and 18 x 10^{-4}M. It will be seen that the inhibition of respiration increased with the concentration of narcotic. This is illustrated more clearly in Fig. 5 where respiration and acetylation are plotted as a function of Nembutal concentration. (In Fig. 5, 100% activity represents 182 ug. of sulfanilamide acetylated, and 475 ul. O_2 taken up in 90 minutes in the absence of Nembutal). As the concentration of Nembutal is increased the inhibition of respiration is likewise increased and tends toward an asymptotic value. The latter is well examplified by the results of Webb and Elliott

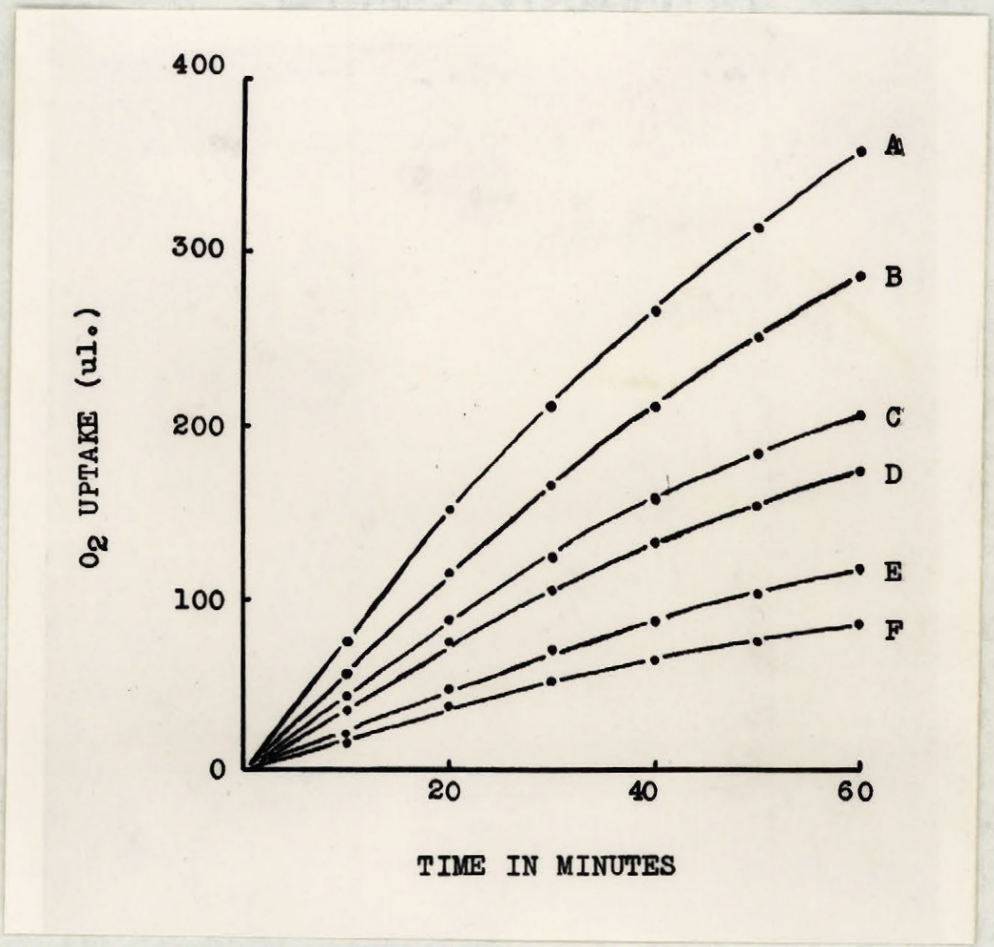


Figure 4.

The effect of varying concentrations of Nembutal on the respiration of a mixture of rat brain homogenate and pigeon liver extract in the presence of pyruvate.

Ordinates: O_2 uptake (ul.).
Abscissae: time in minutes.
Concentration of Nembutal: A, nil; B, 3 x 10^{-4}M; C, 6 x 10^{-4}M; D, 9 x 10^{-4}M; E, 12 x 10^{-4}M; and F, 18 x 10^{-4}M.
Experimental conditions as described in the text.

(150) who found that 15 to 20 per cent of the respiratory activity of rat brain suspensions was resistant to the highest narcotic concentration obtainable, which in this case was Nembutal 0.01M. Thus, the curve for the inhibition of

respiration (Fig. 5) is the type one would expect when the law of mass action is applied. Hence it is apparent that the narcotic inhibits some step or steps in the oxidative chain

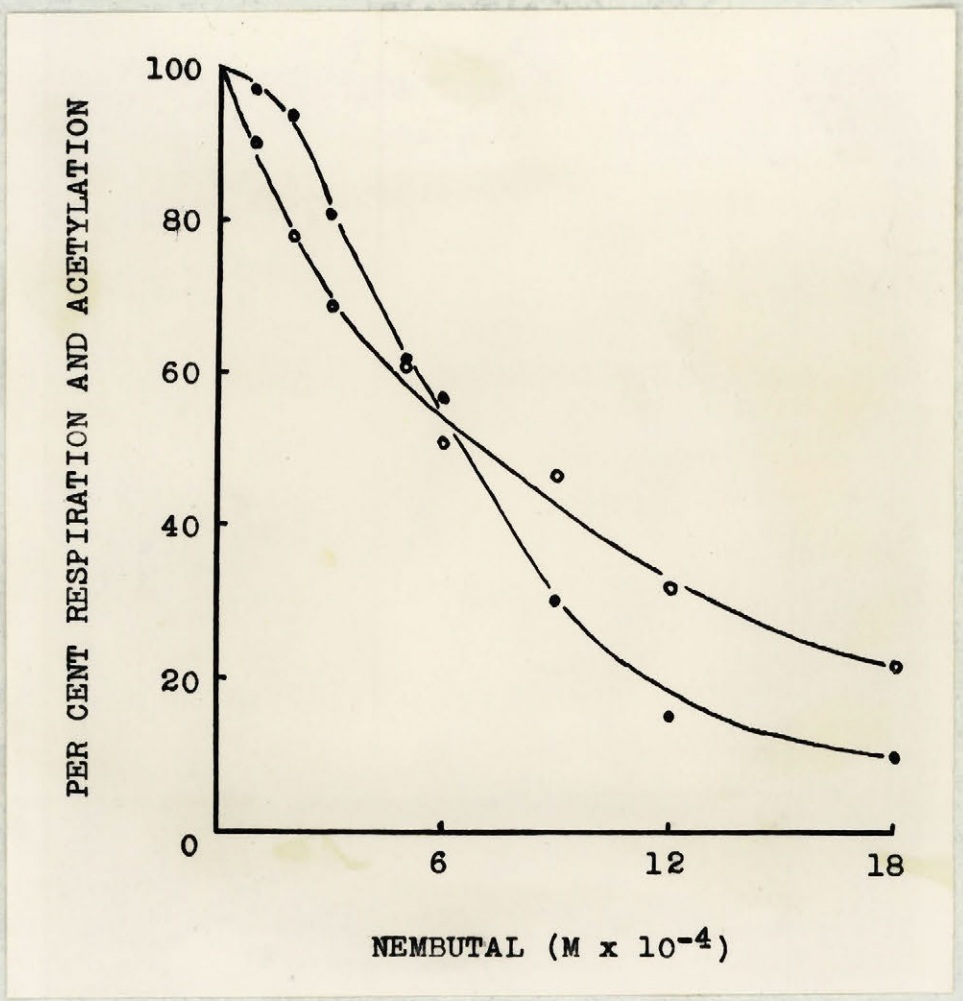


Figure 5.

The relative effects of Nembutal on respiration and sulfanilamide acetylation by a mixture of rat brain homogenate and pigeon liver extract in the presence of pyruvate.

Ordinates: Per cent of normal respiration and acetylation.
Abscissae: Concentrations of Nembutal.
All values plotted represent totals at the end of 90 minutes.
Experimental conditions as described in the text.
. ----- . Acetylation
ϴ ----- o Respiration

according to the law of mass action. The inhibition by Amytal (Fig. 6) presents a similar picture, while with chloretone (Fig. 7) a more direct relationship is observed between narcotic concentration and inhibition of respiration and acetylation.

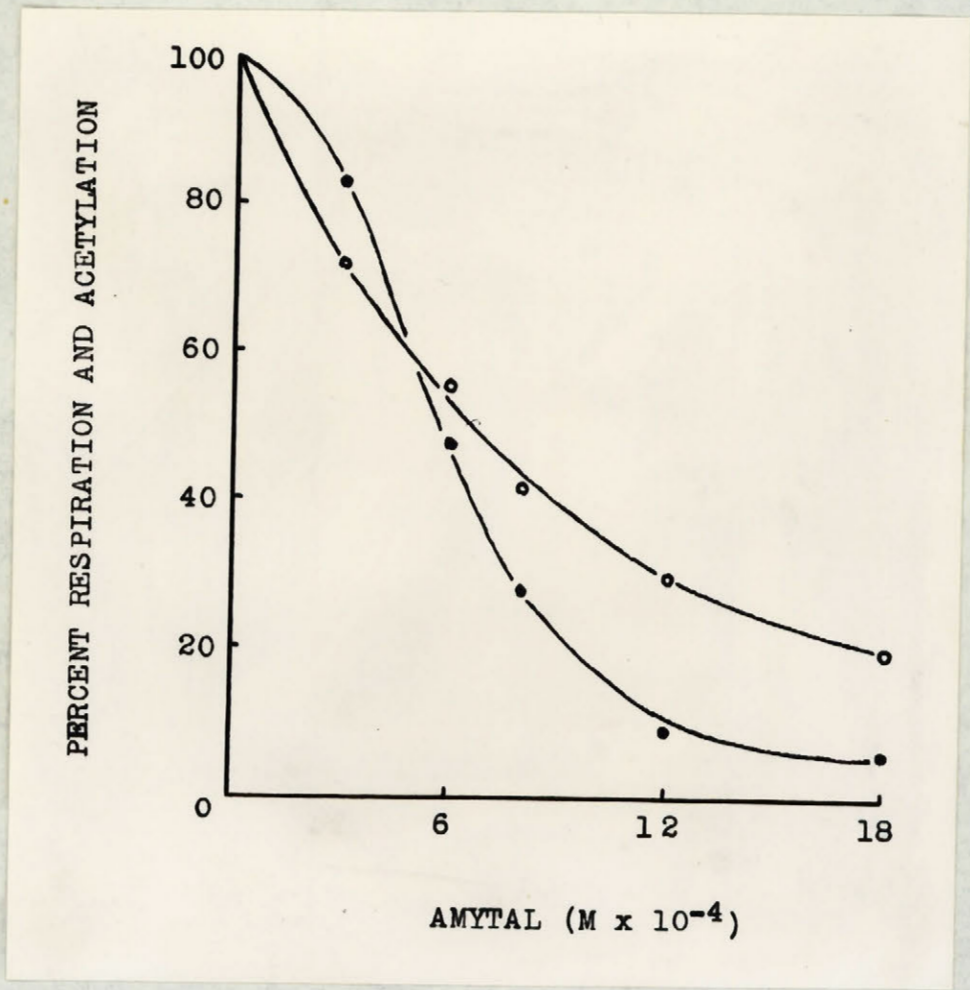


Figure 6.

The relative effects of Amytal on respiration and sulfanilamide acetylation by a mixture of rat brain homogenate and pigeon liver extract in the presence of pyruvate.

Ordinates: Percent of normal respiration and acetylation.
Abscissae: Concentrations of Amytal. All values plotted represent totals at the end of 90 minutes. Experimental conditions as described in the text.
• ------ • Acetylation
o ------ o Respiration

The inhibition of acetylation by Nembutal and Amytal (Fig. 5 and 6, respectively) is of a slightly different character from that of the inhibition of respiration. An examination of the curves indicates that the rate of inhibition of respiration is approximately the same as that of acetylation until the narcotic concentration is reached which inhibits respiration approximately 20%. From this point the rate of inhibition of acetylation is the greater. Now, it is understandable that when respiration is inhibited to only a slight extent the high-energy phosphate generated, although decreased is still adequate to maintain the acetylation process at almost its optimum level. However, a point is reached, at 80% of the normal rate of respiration, when the generation of high energy phosphate is no longer sufficient to meet the requirements for acetylation purposes with the result that a rapid drop in acetylation takes place.

It will be observed that at higher narcotic concentration the acetylation was depressed to a greater extent than was respiration. Such a condition would be expected in view of the evidence that narcotics act at special points on the chain of oxidative events. If a narcotic at high concentration is exercising a large inhibitory effect on an aspect of the respiratory chain that is linked with ATP synthesis, it does not necessarily follow that an equally large inhibitory effect on the total oxygen uptake will also be observed. The steps inhibited by the narcotic may only represent, in terms of total oxygen absorbed by the system, a fraction of the entire process (132,150).

Thus it may be seen that, when the above factors are taken into consideration there appears to be a real relationship between the extent of inhibition of oxidation by a narcotic and that of acetylation.

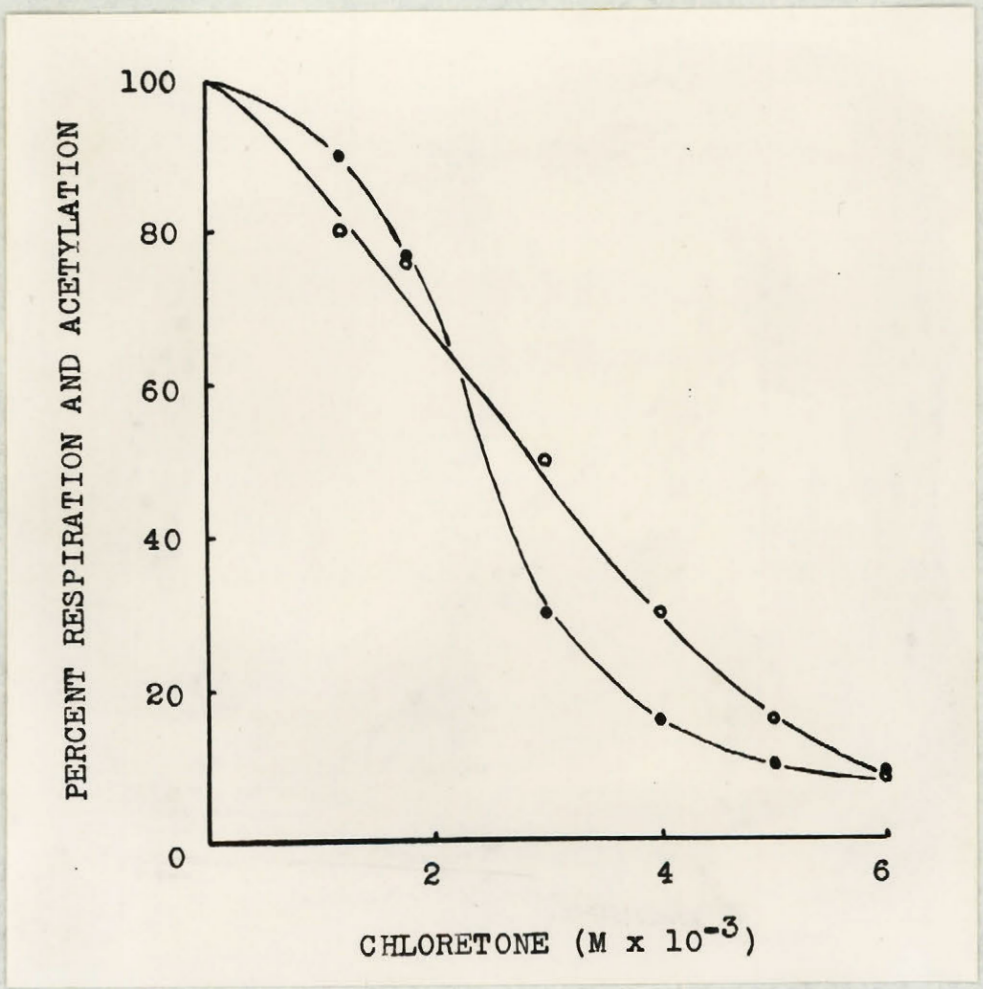


Figure 7.

The relative effects of Chloretone on respiration and sulfanilamide acetylation by a mixture of rat brain homogenate and pigeon liver extract in the presence of pyruvate.

Percent of normal respiration and acetylation.
Abscissae: Concentrations of chloretone. All values plotted represent totals at the end of 90 minutes.
Experimental conditions as described in the text.
. ------ . Acetylation
o ------ o Respiration

The Effect of Hyoscine and Atropine on the Respiration of Guinea Pig Brain Mince

The narcotics employed in the present work consisted mainly of barbiturates and alcohols (chloretone). However, it is known that narcosis can be induced by a wide variety of chemical substances. Therefore, hyoscine and Atropine being entirely different in chemical structure from the barbiturates, were selected as representative substances from the alkaloid group of narcotics for investigation with regard to *in vitro* effects.

Methods

Guinea-pig brain (cerebral hemispheres) was minced by means of a small Latapie mincer; the pulp was suspended in Ca^{++}-free Krebs Ringer-phosphate solution pH 7.4 (214), such that 1 ml. of suspension contained 150 mg. of brain tissue. The manometer flasks contained 1 ml. of brain suspension. Hyoscine and Atropine were dissolved in the same medium and brought to pH 7.4 and added to the manometer flasks to give the concentration indicated in Fig. 8. Glucose was added asan aqueous solution to give a final concentration of 0.017M. 0.2 ml. of 20% KOH was added to the centre-well for CO_2 absorption. The flasks were shaken in the bath for two hours at a temperature of 37°C. Measurements of O_2 uptake were begun after a thermal equilibration period of 5 minutes.

Results

Hyoscine at a concentration of 2×10^{-3}M inhibited the uptake of oxygen by 28%, and at 4×10^{-3}M by 40%. Atropine,

4 x 10^{-3}M, inhibited the uptake of oxygen by 46% (Fig. 8). The degree of inhibition remained constant over a period of two hours, which was the duration of the experiment. The

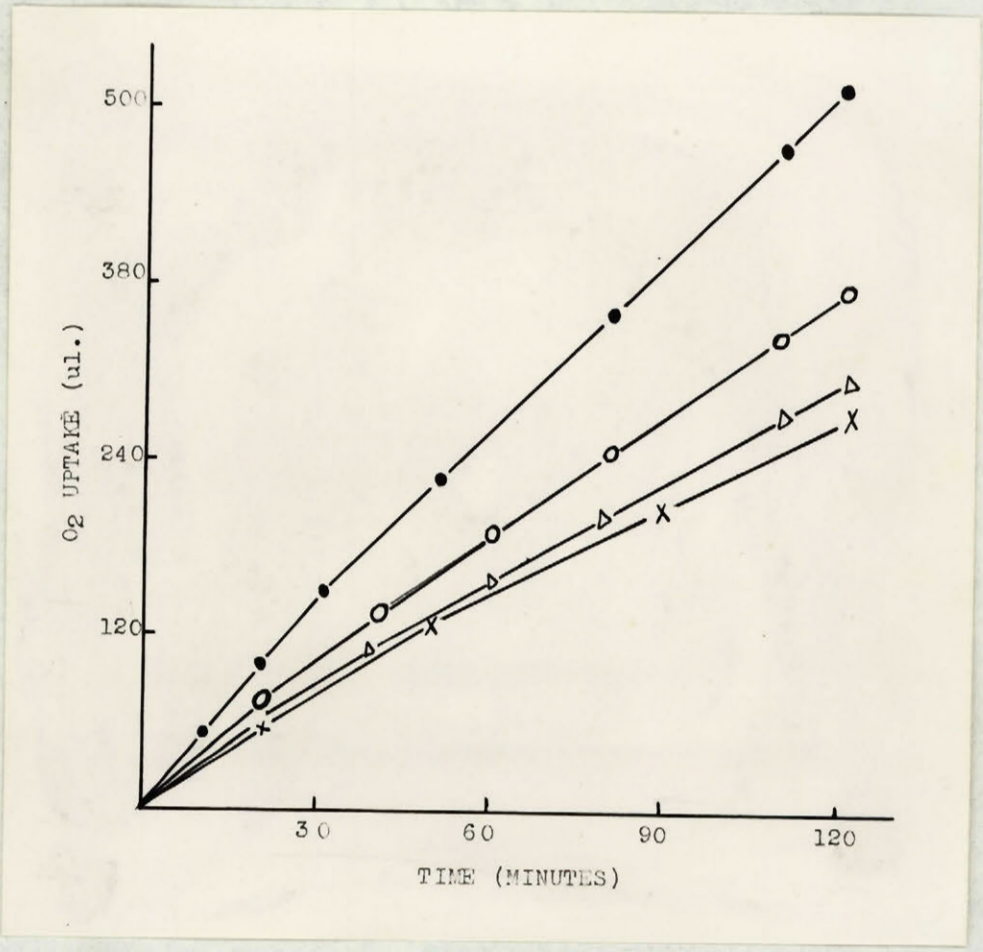


Figure 8.

The inhibition by Hyoscine and Atropine of the O_2 uptake of guinea-pig brain mince respiring in a Ca^{++}-free Ringer-phosphate-glucose medium.

Ordinates: O_2 uptake (ul./150 mg. tissue).
Abscissae: Time in minutes.
Experimental conditions: a s described in the text.
● ------ ● Control (no narcotic)
o ------ o Hyoscine, 2 x 10^{-3}M
△ ------ △ Hyoscine 4 x 10^{-3}M
x ------ x Atropine 4 x 10^{-3}M

results obtained with hyoscine are a confirmation of those obtained by Quastel and Wheatley (133), who found that hyoscine at a concentration of 4 x 10^{-3}M inhibited the respiration of guinea-pig brain slices to the extent of 44%. Thus, it may be seen that both hyoscine and atropine which exhibit narcotic action *in vivo*, are effective in depressing oxygen uptake *in vitro*.

Both hyoscine and atropine at the concentrations shown above had no effect on the acetylation of sulfanilamide by cell-free extracts in the presence of acetate and ATP while aerobically there was an inhibition of sulfanilamide acetylation of 20% and 30% at concentrations of 2 x 10^{-3}M and 4 x 10^{-3}M respectively.

Summary

1. It has been shown that a narcotic (Narconumal) at relatively high concentration had no effect on the formation of acetylcholine by extracts of acetone-dried beef brain.
2. Anaerobic glycolysis in beef brain extracts was not affected by a relatively high narcotic concentration i.e. a concentration which was one and one-half times as high as that concentration which inhibited the respiration of a rat brain homogenate by 76%.

3. Narcotics (Chloretone, Narconumal) inhibited the aerobic acetylation of choline by rat brain mince. The extent of inhibition of acetylation was related to the extent of inhibition of respiration.
4. The addition of ATP was largely effective in overcoming the inhibition by Narconumal and Chloretone of the acetylation of choline in rat brain homogenate.
5. From the results obtained the conclusion was drawn that the narcotics tested do not inhibit the choline acetylating system, but that the inhibition of choline acetylation is due to the suppression of ATP formation as a result of the inhibition of respiration by the narcotic.
6. The narcotics tested (Table XX) did not inhibit, at high concentration, the acetylation of sulfanilamide by cell-free extracts of pigeon liver.
7. Sulfanilamide acetylation was found not to take place in brain tissue preparations.
8. Acetylsulfanilamide was not hydrolyzed by rat brain homogenate under various conditions, as indicated by the complete absence of free sulfanilamide from the medium.
9. A system comprised of a mixture of rat brain homogenate and cell-free extract of pigeon liver was described, which upon the addition of acetate or pyruvate was very active in acetylating sulfanilamide under aerobic conditions.
10. The addition of chloretone at a concentration of 4×10^{-3} M, to the system described in (9) brought about a large

inhibition of respiration and sulfanilamide acetylation. The extent of inhibition of sulfanilamide acetylation was related to the extent of inhibition of respiration.

11. When acetate was the substrated added to the brain-liver preparation, the large inhibition of sulfanilamide acetylation exercised by chloretone was almost overcome by the addition of ATP, while the inhibition of respiration was only slightly relieved or not at all by the addition of ATP. The conclusion was drawn that the inhibition of sulfanilamide acetylation induced by chloretone was a result of the suppression of ATP synthesis, which in turn was due to the inhibition of respiration caused by chloretone.

12. When pyruvate was the substrate in the same preparation the addition of ATP did not relieve the inhibition of sulfanilamide acetylation by chloretone to an appreciable extent. This was interpreted to mean that chloretone inhibited the pyruvic oxidase system, in which case pyruvate could not provide acetate for acetylation purposes; hence, the ineffectiveness of ATP in overcoming the inhibition of sulfanilamide acetylation.

13. It was shown that ferricyanide at a concentration of 0.01M inhibited the acetylation of sulfanilamide by a cell-free extract of pigeon liver to the extent of 75 to 80%. The postulation was made that ferricyanide oxidizes the -SH groups of Coenzyme A.

14. When pyruvate was being oxidized by the brain-liver preparation described in (9), the addition of fumarate to the system gave rise to an inhibition of sulfanilamide acetylation and an increase in the rate of O_2 uptake. It was concluded that the addition of fumarate favoured the formation of citrate at the expense of sulfanilamide acetylation.

15. The addition of malonate reversed the inhibition by fumarate of sulfanilamide acetylation and partially reversed the stimulating effect of fumarate on O_2 uptake. This is consistent with the demonstration that malonate inhibits succinoxidase, which in turn causes an accumulation of intermediates of the tricarboxylic acid cycle, the result being a diversion of Acetyl-CoA towards the formation of acetylsulfanilamide.

16. The addition of succinate alone to the brain-liver preparation was responsible for as high a rate of aerobic sulfanilamide acetylation as that bbtained by the addition of pyruvate alone.

17. It was shown that the one-step oxidation of succinate to fumarate in the presence of a concentration of chloretone which completely blocks the oxidation of fumarate, increased sulfanilamide acetylation, which indicates that succinate oxidation can give rise to utilizable energy for the maintenance of energy requiring metabolic processes.

18. Contrary to the effect of succinate in the presence of chloretone described in (17) above, in the presence of a high concentration of 2,4-DNP succinate did not give rise

to increased sulfanilamide acetylation. The contrasting effects of chloretone and 2,4-DPN in the presence of succinate point to an important difference in the mechanism of action of these drugs.

19. It was shown that the relationship between the extent of inhibition of oxidation and the concentration of narcotic (Nembutal, Amytal and Chloretone) required to bring about the inhibition, indicate a mass action effect of the narcotic on the respiratory process.

20. A real relationship was shown to exist between the extent of inhibition of oxidation by a narcotic and the inhibition of acetylation.

21. It was confirmed that Hyoscine at a concentration of 0.004M inhibits by 40% the respiration of guinea-pig brain tissue. Atropine, at the same concentration inhibits the respiration of guinea-pig brain mince by 46%, and inhibited the aerobic acetylation of sulfanilamide by the brain-liver preparation to the extent of 30%.

AMIDE INHIBITORS OF SULFANILAMIDE ACETYLATION

As a result of the observation that nicotinamide (NAA) could be used effectively as an inhibitor of DPN-ase (diphosphopyridine nucleotidase), (39,184), the addition of NAA became a general practice in the preparation of brain tissue homogenates and extracts. However, in the course of the present work it was found that such preparations inhibited the acetylation of sulfanilamide, when high concentrations of NAA were present. To obtain proof that NAA was responsible for the inhibition, NAA was added to extracts of pigeon liver in the presence of acetate and ATP.

The results are shown in Table XXXIII (Exp. 1 and 2). It may be seen that in the presence of acetate and ATP, pigeon liver extract was capable of a high rate of sulfanilamide acetylation. The addition of NAA, at concentrations normally present in brain homogenates gave rise to a marked inhibition of sulfanilamide acetylation. This effect of NAA was somewhat surprising in view of the fact that no inhibition of choline acetylation was observed when NAA had been used in the brain preparations employed.

The inhibition by NAA increased with concentration, (Table XXXIII, Exp. 2), indicating the likelihood of interaction with enzymes rather than co-factors, taking into consideration the high concentrations of NAA required (approx. 0.02M) to produce an inhibition of 50%.

TABLE XXXIII

THE EFFECT OF NICOTINAMIDE, NICOTINIC ACID, AND CORAMINE ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACTS IN THE PRESENCE OF ACETATE AND ATP

Exp.	Additions	Sulfanilamide acetylated (ug)	Per cent Inhibition of Acetylation
1	Nil	193	---
	NAA 0.02M	112	42
2	Nil	184	---
	NAA 0.005M	153	17
	NAA 0.01M	126	31
	NAA 0.02M	99	46
	NAA 0.035M	70	62
	NAA 0.05M	54	71
3	Nil	176	---
	Coramine (Nikethamide) 0.01M	135	23
4	Nil	190	---
	NAA 0.02M	106	44
	Nicotinic acid* 0.02M	193	0

Experimental conditions: Pigeon liver extract: 1 ml. (≡ 60 mg. per vessel. Acetate 0.02M; ATP 0.0045M; sodium phosphate buffer pH 7.5, 0.02M; $NaHCO_3$ 0.028M; sulfanilamide: 200 ug. Total volume: 3 ml. Gassed with 93% N_2 and 7% CO_2.

Temperature: 37°C. Time: 90 minutes + 15 minutes equilibration.

*Neutralized with NaOH.

The fact that nicotinic acid (0.02M) had no effect on acetylation (Table XXXIII, Exp. 4) indicated, at least, that the amide group was involved in the inhibitory effect of NAA and not the pyridine nucleus.

It was expected that coramine (N,N-diethyl nicotinamide) having no free amide group, would act in the same manner as nicotinic acid, and have no effect on acetylation. However, coramine (0.01M) gave an inhibition of 23% (Table XXXIII, Exp. 3) which was approximately three-fourths that of NAA at the same concentration. There is a possibility that coramine was de-ethylated to nicotinamide (185) and that the latter was responsible for the inhibition of acetylation.

Other amides, varying widely in structure, were tested. Acetamide had no effect (Table XXXIV, Exp. 1), while benzamide proved to be more effective than NAA or semicarbazide (Table XXXV). Adenosine, which is not an amide, but does have an amino group attached directly to a carbon atom of the pyrimidine ring, was half as effective as benzamide at a concentration of 0.02M (Table XXXIV, Exp. 2).

It has been previously shown (Table IX, Exp. 3) that nitrourea has an action similar to 2,4-DNP on the acetylation of sulfanilamide arising from pyruvate dismutation. Nitrourea at a concentration of 0.01M inhibited acetylation in the presence of pyruvate by 40%, while that due to acetate and ATP was inhibited by only 17% (Table XXXIV).

TABLE XXXIV

THE EFFECT OF AMIDES ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT

Exp.	Additions	Sulfanilamide acetylated ug.	Per cent inhibition of acetylation
1	Nil	190	---
	Acetamide 0.02M	191	0
	Adenosine 0.016M	160	17
2	Nil	164	---
	Adenosine 0.02M	121	26
	Benzamide 0.02M	74	55
	Benzenesulfonamide 0.007M	126	12
3	Nil	144	---
	Nitrourea 0.01M	119	17

Experimental conditions: See Table XXXIII.
Acetate 0.02M; ATP: Exp. 1, 0.0045M
Exp. 2, and 3, 0.003M
Sulfanilamide: 202 ug.

Semi-Carbazide

In the course of the investigation of the inhibition by fumarate of sulfanilamide acetylation in cell-free extracts, there were indications that the inhibition was due to oxalacetate formed from fumarate. Semicarbazide was tried as a fixative for oxalacetate in order to overcome the inhibition. However, it was observed that semicarbazide not only failed to relieve the inhibition by fumarate but itself gave rise to an inhibition of sulfanilamide acetylation.

Semicarbazide has been shown by Lipton and Barron (21) to enhance the synthesis of Ach by brain extracts when citrate is used as acetyl-donor and this has been confirmed (Table XIV). Its action in this system is to trap the oxal-acetate which accumulates from the breakdown of citrate, thus preventing an equilibrium from being established.

This effect of semicarbazide may be seen from the results shown in Table XXXV. (It should be pointed out that citrate and acetate were equally effective as acetyl donors for sulfanilamide adetylation when ATP was also present, Exp. 1 and 2. Previously it was shown that citrate alone, or citrate and DPN, did not give rise to acetylation, Table XI, Exp. 1 and 3. However, Stern, et al, 206, have shown that citrate, in the

TABLE XXXV

THE EFFECT OF SEMI-CARBAZIDE ON THE ACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT AT 37°C

Exp.	Additions	Sulfanilamide acetylated ug.	Per cent inhibition of acetylation
1	Citrate 0.02M	91	---
	Citrate 0.02M + semicarbazide 0.02M	52	43
2	Acetate	88	---
	Acetate 0.02M + semicarbazide 0.02M	56	36

Experimental conditions: Pigeon liver extract: 1 ml. (60 mg. powder) sodium phosphate (pH 7.5) 0.02M; ATP 0.002M; $NaHCO_3$ 0.028M. Sulfanilamide added: 105 ug.
Total volume: 3 ml. Gassed with 93% N_2 and 7% CO_2.
Time: 90 minutes + 15 minutes equilibration. Temperature: 37°C.

presence of CoA and reduced DPN, will form Acetyl-CoA). The inhibition of sulfanilamide acetylation by semicarbazide was independent of the acetyl precursor used, whether acetate or citrate, and was of the same order in both cases (Table XXXV, Exp. 1 and 2.).

As an explanation of the inhibition of sulfanilamide acetylation by the amides tested, three possibilities are suggested:

(1) Competitive inhibition of amides with sulfanilamide by the blocking of the active centres of the acetylating enzymes.

(2) Acetylation of the amides, thus competing with sulfanilamide for acetyl-CoA.

(3) Competition with Coenzyme A for attachment to the apoenzyme.

The latter possibility can be ruled out since if such were the case the acetylation of both sulfanilamide and choline would be inhibited by NAA and semicarbazide, inasmuch as both acetylations require Coenzyme A. However, should one point of attachment of CoA to the apoenzyme be by means of the adenine amino group, there is the possibility that adenosine (Table XXVII) could inhibit by competition with CoA. Adenosine could also inhibit by competing for ATP since the phosphorylation of adenosine is known to occur in animal tissues (215).

No attempt has yet been made by means of kinetic studies

to determine whether the inhibition was competitive or non-competitive. Of particular value would be the investigation of NAA in this regard.

Summary

1. The following amides have been found to inhibit the acetylation of sulfanilamide by pigeon liver extracts: Nicotinamide coramine (N,N-diethyl-nicotinamide) semicarbazide, benzamide, and nitrourea.
2. Adenosine, which is not an amide but does have an amino group attached to the pyridine nucleus, also inhibited sulfanilamide acetylation.
3. Nicotinic acid in contrast to nicotamide, had no effect on the acetylation of sulfanilamide.
4. Acetamide, unlike benzamide, had no effect.

THE EFFECT OF SALICYLATE ON THE ACETYLATION OF SULFANILAMIDE

Oral administration of para-aminobenzoic acid, whether as the free acid or the sodium salt has been shown to cause an increase in the plasma level of salicylates in man (200). It has also been shown that para-aminohippuric acid is synthesized by the liver (201). The former investigators suggested that para-aminobenzoic acid depresses the formation of salicyluric acid by forming para-aminohippuric acid, thus competing for glycine.

Although there is evidence that approximately 60% of administered salicylate is excreted in man as salicyluric acid (202), alternative metabolic pathways are known, i.e., formation of the glucuronate (203), and oxidation to gentisic acid (2,5-dihydroxybenzoic acid) (204). Therefore, competition by para-aminobenzoic acid for glycine and consequent depression of salicyluric acid formation may not be the only explanation for the increased plasma levels of salicylate in the presence of para-aminobenzoic acid.

In view of the above considerations it was decided to ascertain the effect of salicylate on the acetylation of sulfanilamide. The aerobic system described on page 139 was used for this purpose.

It may be seen from Table XXXVI that at concentrations of salicylate varying from 1.3×10^{-4} M to 10^{-2} M there was a

stimulation of respiration of 15 to 20% while at the same time the acetylation of sulfanilamide was inhibited by 12% at the lowest concentration of salicylate used, and 93% at a concentration of 10^{-2}M salicylate. Thus, at the latter concentration of salicylate the inhibition of sulfanilamide acetylation was almost complete. However, 6×10^{-3}M salicylate inhibited the

TABLE XXXVI

THE EFFECT OF SALICYLATE ON THE AEROBIC ACETYLATION OF SULFANILAMIDE BY A MIXTURE OF RAT BRAIN HOMOGENATE AND PIGEON LIVER EXTRACT (at 37°C)

Exp.	Additions	O_2 uptake µl.	O_2 uptake percent of control	Sulfanilamide acetylated µg.	Acetylation percent of control
1	Nil	642	--	148	--
	Sodium salicylate 1.3×10^{-4}M	740	115	130	88
	Sodium salicylate 4×10^{-3}M	766	119	90	61
2	Nil	696	--	188	--
	Sodium salicylate 3.3×10^{-3}M	843	120	108	57
	Sodium salicylate 1×10^{-2}M	828	119	13	7
3	Nil	659	--	153	--
	2,4-DNP 4.5×10^{-4}M	540	82	18	12
	2,4-DNP 7×10^{-4}M	527	80	11	7

Exptl. Conditions: see page 138. Time: 90 minutes.
Substrates added: sodium pyruvate 0.02M; sodium fumarate 0.006M.

anaerobic acetylation of sulfanilamide in the presence of acetate and ATP only to the extent of 12%, i.e. the amount of sulfanilamide acetylated in 90 minutes was reduced from 171 ug.

to 151 ug., indicating that salicylate was not competing with sulfanilamide for Acetyl-CoA, and did not interfere with ATP utilization to an appreciable extent.

In the light of the above data it appears that salicylate interfered with the production of high-energy phosphate under aerobic conditions, and in this respect resembles the effect of 2,4-DNP, i.e. the "uncoupling" of phosphorylation from oxidation, inasmuch as respiration was stimulated even at 10^{-2}M salicylate concentration, and acetylation was inhibited by 93%. (It should be mentioned that Lutwak-Mann (205) found no appreciable inhibition of the respiration of rat liver slices by 5 x 10^{-2}M salicylate, but appreciable inhibition did occur at 0.1M concentration). However, a comparison of the results of Exp. 2 and 3 (Table XXXVI) will show that 2,4-DNP is more than 14 times as effective as salicylate in depressing the acetylation of sulfanilamide. Whereas 2,4-DNP at a concentration of 7 x 10^{-4}M depressed the acetylation of sulfanilamide to 7% of the control level, salicylate at a concentration of 10^{-2}M was required to produce the same effect.

The possibility that salicylates act by depressing the formation of high energy phosphate is a matter for consideration. It would explain the increase obtained in the plasma level of salicylates by the oral administration of para-aminobenzoic acid. The formation of para-aminohippuric has been shown to require ATP (201). It is likely that the formation of salicyluric acid also requires ATP. A diminution in ATP formation by the liver as a result of the presence of

salicylate would create a condition of ATP deficiency. Therefore, competition by para-aminobenzoic acid for ATP rather than glycine, with a consequent drop in salicyluric acid formation may account for the increased plasma level of salicylates. There is also the fact that the acetylation of para-aminobenzoic acid would represent a further drain on the supply of ATP.

Summary

1. Salicylate, at a concentration of 10^{-2} M, stimulated the respiration of a mixture of rat brain homogenate and pigeon liver extract by approximately 20%, but at the same time inhibited the aerobic acetylation of sulfanilamide in the same system by 93%.
2. Salicylate has no appreciable effect on the acetylation of sulfanilamide by pigeon liver extract in the presence of acetate and ATP.
3. The effect of salicylate bears some resemblance to that of 2,4-DNP (Table XXXVI), although the former must be used in much higher concentration to produce the same effect.
4. It is therefore suggested that salicylate may produce its effects by suppressing ATP formation in a manner similar to 2,4-DNP, i.e. by an "uncoupling" of phosphorylation from respiration.

FLUOROACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACTS

Recent evidence indicates that the inhibition of respiration caused by fluoroacetate is due to the accumulation of fluorocitrate, a non-metabolite (197,198). Chenoweth (138) has reported an increase in the acetylation of sulfanilamide and of p-aminobenzoic acid by rabbit liver in the presence of 0.02M fluoroacetate; and also that the formation of acetylcholine from choline in the presence of glucose or pyruvate was not affected by 0.02M fluoroacetate. Therefore, the conclusion was drawn by Chenoweth that the increased acetylation of foreign amines is the result of an inhibition of acetate metabolism in consequence of which more acetate becomes available for acetylations.

Now, it would appear that since fluoroacetate can readily participate in the formation of fluorocitrate, it might also participate in the formation of fluoroacetylcholine and fluoroacetylsulfanilamide. Therefore, it was decided to test the ability of fluoroacetate to behave as a precursor of the latter compound.

Pigeon liver powder, prepared as described in "Experimental Methods" was extracted in the manner already described with 0.15M KF. 1 ml. of pigeon liver extract (equivalent to 60 mg. of powder) was added to each manometer flask. In addition the flasks contained sodium phosphate buffer, pH 7.5,

0.02M; $NaHCO_3$ 0.028M; ATP 0.003M; sulfanilamide 203 ug., and other additions as indicated in Table XXXVII. (All concentrations are final, in a total volume of 3 ml.). The vessels were gassed for 10 minutes with 93% N_2/ 7% CO_2, and incubated at 37°C for 90 minutes.

TABLE XXXVII

FLUOROACETYLATION OF SULFANILAMIDE BY PIGEON LIVER EXTRACT

Additions	Sulfanilamide acetylated (ug)
Nil	14
Acetate 0.02M	176
Fluoroacetate 0.01M	176
Acetate 0.02M + Fluoroacetate 0.01M	182

Experimental conditions as described in the text.

The results shown in Table XXXVII seem to indicate that fluoroacetate can participate in acetylation reactions just as efficiently as acetate itself. On the basis of known facts such a result could be predicted. However, the fluoroacetate used for this work may have contained acetate as an impurity, although it is unlikely that acetate could be present as an impurity in high enough concentration to give the result shown in Table XXXVII. In any case, proof of the formation of fluoroacetylsulfanilamide will depend upon

isolation of the product formed and demonstration of the presence of fluoride in the molecule.

Elliott and Kalnitsky (105) found that citrate oxidation was inhibited to the same extent as the oxidation of acetate when washed rabbit kidney cortex was incubated with fluoroacetate and oxalacetate for 15 minutes before the addition of citrate. The inhibition was shown to be competitive. However, under the same conditions the oxidation of cis-aconitate and iso-citrate was not inhibited. These facts indicate enzymic inhibition on the part of fluorocitrate.

There is a remarkable similarity of fluoroacetate induced convulsions in rhesus monkeys and in man to a grand mal epileptic seizure (138). Electrical activity of the cortex reaches very high potentials during fluoroacetate poisoning but can be obliterated by barbiturates and anticonvulsants (139). The sensitivity of rats to electrically-induced convulsions is increased about ten times by fluoroacetate (199). In the light of these observations there is a possibility that fluoroacetate affects the central nervous system by the formation of fluoroacetylcholine which may conceivably be more resistant to attack by cholinesterase than is acetylcholine. Alternatively, fluoroacetylcholine may inhibit cholinesterase and thus prevent the hydrolysis of acetylcholine, in much the same manner as DFP, or TEPP.

GENERAL DISCUSSION AND CONCLUSIONS

When the investigation of the effects of narcotics on biological acetylations was begun the view was taken, as a working hypothesis, that the depression of cellular function which characterizes the narcotic effect is the result of the suppression of oxidative ATP formation upon which metabolic processes depend. To account for the diverse effects of narcotics on a variety of living systems it was apparent that a fundamental process, common to all living matter, must be affected by narcotics. The experimental results which have been described are consistent with the above point of view.

It was shown that an inhibition of respiration is followed by a concomittant drop in acetylation, and that the addition of ATP to the aerobic system alleviates, to a large extent, the narcotic effect on acetylation. This effect of added ATP indicates that the narcotic inhibition of acetylation is the result of a suppression of oxidative synthesis of ATP. Furthermore, the experimental data show that narcotics at high concentration do not exercise a general effect on enzyme systems as a whole, but that the effect is somewhat specific, being more or less restricted to a limited section of the respiratory process, while acetylating enzymes *per se*, the pyruvate dismutation system, and the glycolytic sequence in beef brain extract are not affected.

Although there is as yet no direct proof that the suppression of ATP synthesis which accompanies the inhibition of respiration is responsible for the symptoms by which the narcotic state is recognized, there are indications that this may be so. There is clear evidence that during narcosis in man and animals there is a fall in the respiratory activity of the nervous system (218,219,220). In this connection it should be remembered that the over-all drop in the uptake of oxygen by the brain does not give a true picture of what may be taking place at a particular centre in the brain. Thus, a large drop in respiration may take place at a particular centre more sensitive to the narcotic than the large mass of brain tissue. The effect of the narcotic at this centre may have widespread ramifications, and yet the total drop in oxygen uptake by the brain may not be great in relation to the observed narcotic effects.

The importance of acetylcholine in nerve physiology is well-known. A depression of respiration at a particular centre of the nervous system may well result in a decreased acetylcholine synthesis and thus impede the transmission of nervous impulses. Thus, the inhibition of acetylcholine synthesis may be a contributing factor in narcosis. To illustrate, McLennan and Elliott (130) observed that a low concentration of Nembutal which inhibited the oxygen consumption of brain slices by 15 to 20% caused a 50% decrease in the synthesis of acetylcholine.

The view is widely held that narcosis is due to the effect of the narcotic drug on the cellular membrane of the nerve cell. Narcotics supposedly alter the permeability of the nerve cell which leads to inability to transmit the nerve impulse. However, there is conflicting evidence on the effects of narcotics on permeability. The narcotics in many cases appear to alter the structure of plasma membranes and increase or decrease their permeability. It is quite possible that the effect of narcotics on the permeability of the nerve cell may be of importance in narcosis, but at the same time it must be remembered that cellular permeability is dependent on energy factors, and change of permeability may be the result of energy changes in the cell membrane. Thus, the cellular membrane theory of narcosis is not incompatible with the view that inhibition of ATP formation is responsible for the narcotic state.

The contrasting effects of narcotics and dinitrophenol on the acetylation of sulfanilamide by the pyruvate dismutation system of pigeon liver extract, and on the acetylation associated with the oxidation of succinate illustrates the difference in the mechanism of action of these two types of drugs. It was shown that narcotics had no appreciable effect on the acetylation of sulfanilamide by pigeon liver extracts in the presence of pyruvate and DPN, while 2,4-dinitrophenol inhibited the acetylation markedly. At the same time, 2,4-dinitrophenol did not inhibit the acetylation of sulfanilamide

when acetate and ATP were added as acetylating agents. Also, it was found that the oxidation of succinate, in the presence of a narcotic concentration which completely inhibited fumarate oxidation, gave rise to appreciable acetylation of sulfanilamide; when the narcotic (chloretone) was replaced by 2,4-dinitrophenol the oxidation of succinate was associated with no acetylation of sulfanilamide. Whereas narcotics have no effect on anaerobic reactions taking place in cell-free extracts, dinitrophenol affects phosphorylations taking place in both aerobic and anaerobic systems. This indicates that narcotic effects are associated with respiratory processes and that the action of dinitrophenols is not restricted to phosphorylations coupled to respiratory processes. That is not to say that a clear-cut differentiation necessarily obtains between narcotics and dinitrophenol-like compounds. There may be an overlapping in some cases; i.e. certain substances which have narcotic action may also be found to exhibit an uncoupling effect. Hyoscine may be taken as an example (Table X, page 73).

The value of the use of succinate as an analeptic is still a matter of doubt. The fact that the oxidation of succinate in the presence of a high narcotic concentration can give rise to sulfanilamide acetylation <u>in vitro</u> indicates that there is a sound basis for continued investigation into the conditions under which succinate administration might relieve a narcotic state.

It was mentioned above that 2,4-dinitrophenol had a marked inhibitory effect on the acetylation of sulfanilamide

arising from the dismutation of pyruvate by pigeon liver extracts. This effect of 2,4-dinitrophenol indicates that phosphorylation is associated with the dismutation of pyruvate. There are other indications that esterification of inorganic phosphate takes place in the course of the reaction. Firstly, there is the fact that sulfanilamide acetylation does not occur in the absence of phosphate, i.e. sulfanilamide cannot replace orthophosphate in the pyruvate dismutation reaction which takes place in crude pigeon liver extracts. This implies that phosphate may play an essential role in the formation of acetyl-CoA. Secondly, the stimulating effect of ATP, ADP, and AMP (muscle adenylic acid) on sulfanilamide acetylation (Table VIII, page 65) indicates that ATP is, under some circumstances, implicated in the formation of Acetyl-CoA from pyruvate. Should pyruvate break down directly to Acetyl-CoA it is difficult to account for the effect of ATP on acetylation. The increased acetylation obtained with ADP is due to the formation of ATP through myokinase activity. The stimulating effect of AMP on sulfanilamide acetylation in the presence of pyruvate and DPN seems to represent strong evidence for phosphorylation coupled with pyruvate dismutation. The conditions are present in pigeon liver extracts for the phosphorylation of AMP. Catalytic amounts of ADP are present to act as primary phosphate acceptor. The presence and activity of myokinase in pigeon liver extracts are indicated by the fact that ADP can give rise to sulfanilamide acetylation in the presence of acetate. Since AMP brings about no increase in

acetylation in the presence of acetate, it is apparent that AMP is phosphorylated in the presence of pyruvate and DPN with a consequent increase of high-energy phosphate in the system.

Hunter and Hixon (217) found no effect of dinitrophenol on the phosphorylation associated with the anaerobic dismutation of α-ketoglutarate. Also, under aerobic conditions one of the phosphorylations coupled to α-ketoglutarate oxidation was found to be unaffected by dinitrophenol. This fact, in conjunction with the lack of effect of dinitrophenol on the anaerobic dismutation indicated that the dinitrophenol resistant phosphorylation was at the substrate level. The effect of 2,4-DNP on pyruvate dismutation may mean that the phosphorylation associated with the dismutation of pyruvate takes place during the oxidation of $DPNH_2$ by pyruvate, rather than during the oxidation of the substrate itself. Although no direct evidence can be offered for this postulation it might be regarded as a possibility.

It was pointed out above that sulfanilamide could not substitute for orthophosphate in the pyruvate dismutation taking place in crude pigeon liver extracts. Korkes et al (84) have demonstrated that orthophosphate is not an obligatory reactant in the anaerobic oxidation of pyruvate by soluble enzyme preparations from E. coli and S. faecalis. The authors observed that oxalacetate could replace ortho-

phosphate in the dismutation reaction providing the enzymes for citrate formation were also present (Reaction 4, page 45), and concluded that pyruvate yields Acetyl-CoA without the intermediary formation of acetylphosphate. Furthermore, Korkes, et al (216) duplicated the E. coli results with soluble enzyme preparations from pig heart, in which they demonstrated the formation of acetylphosphate from pyruvate on the addition of bacterial transacetylase to the pig heart system. The authors have claimed that the results obtained with the pig heart preparation prove the general validity of the results obtained with the E. coli enzymes.

Now, if it is true both for animal and bacterial tissues that pyruvate yields Acetyl-CoA directly, the addition of sulfanilamide along with the appropriate enzymes should substitute for orthophosphate as an acetyl acceptor in the same manner as oxalacetate (Reaction 4, page 45). Furthermore in the presence of pyruvate and DPN, sulfanilamide acetylation would be expected to occur in pigeon liver extract in the complete absence of orthophosphate, since sulfanilamide can act as an acetyl acceptor for the acetyl-CoA formed from pyruvate. However, experimental results shown on page 61 indicate that sulfanilamide cannot substitute for orthophosphate since negligible acetylation occurred in the absence of phosphate. When phosphate was added to the pigeon liver extract an active acetylation of sulfanilamide took place and the CO_2 output was greatly increased. It is evident that the

dismutation reaction as formulated by Korkes et al (216) is not consistent with the experimental results presented here. Further proof of the general validity of the results obtained by Korkes et al with the E. coli enzymes seem to be required.

Stern et al (206) have shown that citrate, like pyruvate, can form acetyl-CoA directly, i.e., without the intervention of ATP, in the presence of $DPNH_2$ and CoA. The enzymatic breakdown of citrate to form acetyl-CoA can also take place in the presence of a DPN-linked dismutation reaction, such as the alcohol dehydrogenase system. The addition of citrate to the pyruvate dismutation system of pigeon liver extract (Table XI, page 75) increased the acetylation of sulfanilamide by 100%, which is consistent with the observations of Stern, et al. It was also shown that citrate, in the presence of ATP, is an effective acetyl precursor for the acetylation of sulfanilamide (Table XI) and choline (Table XIV). These facts indicate that ATP can participate in the formation of acetyl-CoA from citrate. ATP participation in the formation of acetyl-CoA is also implied in the effects of adenylic compounds on sulfanilamide acetylation in the presence of pyruvate.

CLAIMS TO ORIGINAL RESEARCH

1. The stimulation by pyruvate of the anaerobic glycolysis of glucose in a beef brain extract is accompanied by an increased synthesis of acetylcholine. This is in contrast to the inhibitory effect of pyruvate on acetylcholine synthesis under other conditions.
2. Pyruvate dismutation has been found to take place in beef brain extracts under anaerobic conditions.
3. The dismutation of pyruvate by beef brain extracts gives rise to an increased rate of acetylcholine synthesis.
4. Fumarate has no effect on the dismutation of pyruvate by beef brain extracts but inhibits acetylcholine synthesis markedly.
5. The dismutation of pyruvate by cell-free extracts of pigeon liver is associated with a large increase in the rate of sulfanilamide acetylation. The dismutation requires orthophosphate, which cannot be replaced by sulfanilamide.
6. In the presence of pyruvate and DPN the addition of ADP or AMP (muscle adenylic acid) to a crude cell-free extract of pigeon liver results in a large increase in the rate of sulfanilamide acetylation. When acetate is used as the source of acetyl, AMP has no effect on sulfanilamide acetylation.
7. Pigeon liver extracts contain myokinase, which is not inhibited by 0.05M KF.

8. 2,4-Dinitrophenol, nitrourea, and to a lesser extent, hyoscine, inhibit the sulfanilamide acetylation which is associated with pyruvate dismutation, but have no effect on acetylation when acetate and ATP are used as acetylating agents.

9. Narcotics (barbiturates, and chloretone) at high concentration have no effect on the acetylation of sulfanilamide by cell-free extracts of pigeon liver, nor do they affect anaerobic glycolysis and acetylcholine synthesis taking place in extracts of acetone-dried beef brain.

10. Narcotics (chloretone, nembutal) inhibit the aerobic acetylation of choline taking place in rat brain mince.

11. The addition of ATP is largely effective in relieving the inhibition of choline acetylation caused by chloretone and narconumal under aerobic conditions.

12. Narcotics markedly inhibit the respiration and aerobic acetylation of sulfanilamide taking place in a mixture of rat brain homogenate and pigeon liver extract, whether the added source of acetyl be pyruvate or acetate.

13. The addition of ATP is largely effective in relieving the narcotic inhibition of sulfanilamide acetylation taking place in a mixture of rat brain homogenate and pigeon liver extract under aerobic conditions, when acetate is the added source of acetyl. At the same time the addition of ATP has little effect on the inhibition of respiration.

14. The inhibitory effect of fumarate, malate, and oxalacetate on sulfanilamide acetylation by extracts of pigeon liver can be overcome by the addition of neutral cyanide, which at a concentration of 0.1M does not inhibit sulfanilamide acetylation taking place in pigeon liver extracts.

15. Bayer-205 (suramin) at a concentration of 3.5×10^{-4}M completely inhibits sulfanilamide acetylation by pigeon liver extracts.

16. Neither the acetylation of sulfanilamide nor the hydrolysis of acetylsulfanilamide takes place in brain tissue.

17. The addition of fumarate inhibits the aerobic acetylation of sulfanilamide when pyruvate is the source of acetyl. The fumarate inhibition is relieved by the addition of malonate.

18. The one-step oxidation of succinate to fumarate in the presence of a concentration of chloretone which completely blocks the oxidation of fumarate, gives rise to increased sulfanilamide acetylation, which indicates that succinate oxidation can give rise to utilizable energy for the maintenance of energy requiring metabolic processes.

19. The following amides inhibit the acetylation of sulfanilamide by cell-free extracts of pigeon liver: nicotin-

amide, coramine (N,N-diethylnicotinamide), semicarbazide, benzamide, and nitrourea. Adenosine, and ferricyanide also inhibit. Nicotinic acid and acetamide have no effect on sulfanilamide acetylation.

20. Sodium salicylate at a concentration of 10^{-2} M stimulates respiration of a mixture of rat brain homogenate and pigeon liver extract, while at the same time inhibiting the aerobic acetylation of sulfanilamide by 93%. Salicylate has negligible effect on sulfanilamide acetylation by pigeon liver extract in the presence of acetate and ATP. The effect of salicylate bears some resemblance to that of 2,4-dinitrophenol.

21. The addition of fluoracetate and ATP to a pigeon liver extract causes the disappearance of added sulfanilamide from the medium, indicating the formation of fluoroacetylsulfanilamide.

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