PURIFICATION OF ACETATE IN PLANT TISSUE SUSPENSIONS

by James L. Webb

THE FORMATION OF ACETATE IN BRAIN TISSUE SUSPENSIONS

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Introduction

Although acetic acid has been found to be produced by brain tissue, it has also been found to be metabolically inert when added to brain slices and suspensions. That brain tissue, which carries on the synthesis of acetylcholine as a phase of its functional activity, can have no use for added acetate, was thought to be an interesting paradox.

Carbohydrate is the main, if not the only, source of metabolic energy in brain tissue, and the acetate formed by brain tissue preparations is apparently derived from intermediary products of carbohydrate metabolism. It was felt that investigation of the conditions under which acetate is produced in brain tissue, and the factors which effect this production, might throw light on the role of acetate in brain metabolism. In view of the considerable attention which is at present being given to acetate, or some reactive two-carbon compound of apparently central significance in the metabolism of carbohydrate, fatty acids and some amino acids, this study seemed timely.

Acetic Acid in Brain Metabolism

Brain cortex slices, from guinea pigs, under anaerobic conditions, were found by Krebs and Johnson (33) to produce acetate from pyruvate via a dismutation reaction, yielding one molecule each of acetic acid, lactic acid, and carbon dioxide for every two molecules of pyruvate reacting, accounting for 30% of the pyruvate utilized under these conditions. This reaction is believed to represent the main anaerobic pathway of pyruvate

metabolism. This dismutation of pyruvate was not found to be inhibited by acetate, fumarate, glutamate, or malonate.

Elliott et al (17,18,19) and later Weil-Malherbe (83) also showed the anaerobic dismutation of pyruvate by brain tissue although the activity found by these authors was considerably lower than that reported by Krebs and Johnson.

Long (48) found that pigeon brain minces when incubated aerobically in the presence of pyruvate formed considerable amounts of acetate. He estimated that about 25% of the pyruvate used was converted to acetate and 80% of this acetate production occurred by direct oxidation of the pyruvate. Long estimated the amount of pyruvate used from his oxygen uptake figures, applying certain assumptions, and did not actually determine the amount of pyruvate used.

Elliott et al (20) in similar experiments on aerobic, isotonic, rat brain suspensions, found that only 14% of the pyruvate used by these preparations when incubated with pyruvate, went to acetic acid. The amount of lactate formed (12% of the total amount of pyruvate used), was close enough to the acetate figures to account for all of the acetate by a dismutation of pyruvate. These authors consider that the direct oxidation, and dismutation reactions, are not fundamentally dissimilar, but merely depend on whether other molecules of pyruvate, or other substances, can accept the hydrogen made available by the oxidation of pyruvate to acetate and carbon dioxide These investigations indicated that acetate is formed from pyruvate in appreciable quantities both aerobically and anaerobically and by probably the same mechanism in both cases.

The acetate which is formed and yet not utilized, by brain tissue in the intact animal, is probably swept away by the blood and used in some other tissue by way of one of the pathways presented in the following survey of the biochemistry of acetate or the acetyl group.

Role of Acetate in Intermediary Metabolism

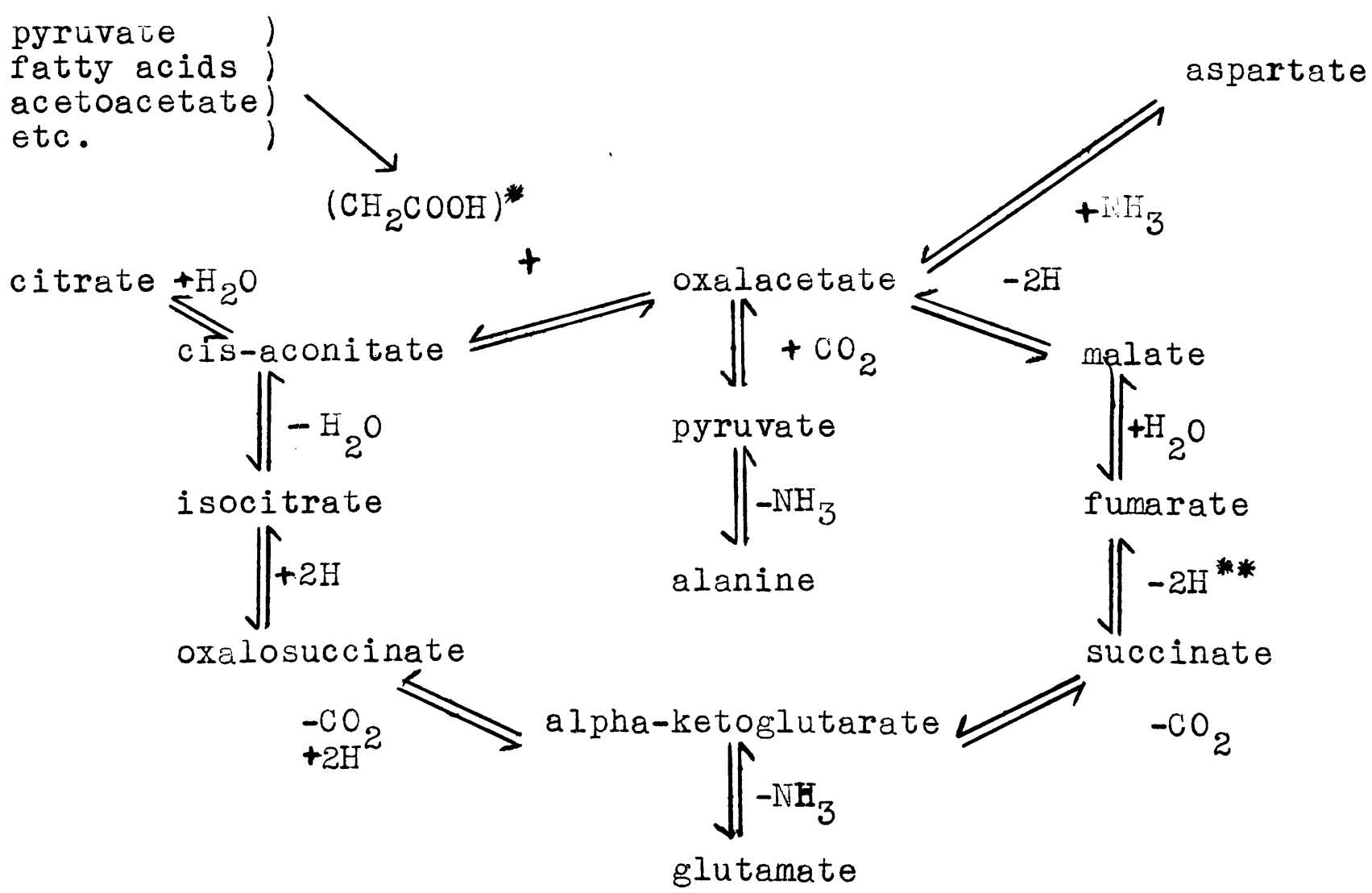
"In vivo" acetate metabolism has been studied by Smyth (74) who found that intravenously injected acetate was rapidly metabolized. Acetate excretion in the urine only accounted for a small fraction of the acid disappearing from the blood. The liver was found to be the most important organ in the utilization of acetate, accounting for about 50% of the acetate used, while the kidney was found to be no more important than other extra-hepatic tissues in the metabolism of acetate. A nephrectomized and evicerated animal could metabolize acetate at a rate of about one-half that of the intact animal. Acetate consumption observed in the intact cat following intravenous acetate injection was 9.3 millimoles per kilogram per hour. That acetate fed to rats is quickly used, was also found by Buchanan et al (9).

Some of the acetate fed to animals is used for the synthesis of liver glycogen, for Lorber, Lifson, and Wood (51), found that when acetate containing isotopic carbon in both carbons of the molecule, was fed to rats, the isotope appeared in the carbon of liver glycogen. The distribution of the isotope was scattered, the amounts of heavy carbon found in positions 1, 2, 5, and 6 were almost equal to the amounts found in positions 3 and 4. These latter positions would be the ones which would

contain all, or nearly all, of the isotope, if the incorporation of the heavy carbon had been due solely to decarboxylation of the acetate and refixation of the carbon dioxide evolved. From these findings it was concluded that the acetate molecule as a whole is used in the synthesis of liver glycogen in the intact rat, and that carbon dioxide fixation does not play the major part in the incorporation of acetate carbon in liver glycogen.

The incorporation of acetate into glycogen and other biological materials, as well as its complete oxidation to CO_2 and water, probably involves an oxidative cycle of tissue metabolism, the scheme for which was originally proposed by Krebs, and has been elaborated by him (34) and many other workers. This scheme is outlined below.

<u>The Krebs Tricarboxylic Acid Cycle</u>



* Represents "active acetate".
** Malonate blocks the cycle here.

Acetate presumably takes part in this cycle in the form of hypothetical, extremely reactive, two-carbon fragment, "active acetate", which is not, however, acetic acid itself. The acetate molecule is probably converted into this active form at the site of reaction in tissues which can use free acetate, this fragment then takes part in biological processes.

Rittenberg and Bloch (71) have shown that acetate does pass through the Krebs cycle "in vivo". The isotope of labelled acetate, when fed to rats, was found in the aspartic and glutamic acids isolated from the body protein of the rats after death. These two amino acids are in equilibrium with oxalacetic and alpha-ketoglutaric acids, respectively, which are members of the Krebs cycle. Liver proteins were found to be especially rich in isotope content, and the amounts of isotope found were too large to be accounted for wholly by the carbon dioxide fixation mechanism.

"In vitro" experiments have shown that acetate and acetoacetate, a derivitive of fatty acid metabolism, are oxidized by means of the Krebs cycle. Buchanan et al (10) have shown that both of these compounds are oxidized by way of the Krebs cycle in suspensions of guinea pig kidney cortex. When these compounds were labelled with isotopic carbon and incubated with the kidney preparations, the isotope was later found to have been incorporated into alpha-ketoglutarate, fumarate, and succinate. The mechanism of carbon dioxide evolution and subsequent refixation could not have accounted for the transfers of isotope, and since the isotope found in the alpha-ketoglutarate was almost wholly in the "gamma" carbon of that compound, citric acid was excluded as an intermediate. Citric acid is

a symmetrical compound so that if it had been an intermediate, the isotope could not have been concentrated in one carbon only. When acetate was added to these tissue preparations, approximately 70% of the acetate utilized passed through the Krebs cycle.

Weinhouse et al (87,88) have shown that liver and kidney slices are able to condense acetate to acetoacetate. Their evidence seems to show that the acetate is condensed with an "active" form of acetate, since only about 45% of the acetoacetate carbon had come from the isotopic acetate added to the preparations. This scheme of acetate condensation was also put forward by Davies (15) and by Svenseid et al (80) who found that the labelled carbon of fed acetate appeared predominantly in only one half of the acetoacetate found, the half which contained the carboxyl group was found to contain nearly all of the isotopic carbon.

Lehninger (35,36) using rat liver enzyme suspensions with added adenosine triphosphate, found that the oxidation of octanoate by this preparation resulted in the formation of two molecules of acetoacetate. In the presence of malonate, two molecules of pyruvate can be oxidized by this preparation to give one acetoacetate molecule plus two molecules of carbon dioxide. When fumarate and malonate were added, acetoacetate production was decreased and citrate accumulated. It was concluded that reactive fragments, probably two carbon, which are produced on oxidation of either pyruvate or fatty acid, may react together to give acetoacetate, or with oxalacetate (from fumarate) to give citrate. None of a series of known four

carbon or two-carbon compounds, including acetate, acetyl phosphate, and acetyl pyrophosphate, was found to be involved in the formation of acetoacetate.

With liver preparations similar to those of Lehninger, Crandall et al (14) using pyruvate with both alpha and beta carbons isotopic, found that the acetoacetate formed by the enzyme suspensions in the presence of pyruvate contained the isotope equally distributed among all four carbons. The acetoacetate therefore came solely from the two carbons of pyruvate which were isotopically labelled.

Acetate itself was found not to give rise to acetoacetate but if carboxyl-labelled acetate was added along with normal pyruvate, the acetoacetate formed was found to contain equal amounts of the isotope in the carboxyl and carbonyl groups. In this case the added acetate must have condensed with two-carbon fragments from pyruvate oxidation to give an equimolar mixture of carbonyl and carboxyl labelled acetoacetate.

Weinhouse et al (19) found that if acetate, containing heavy carbon, was incubated with rat liver slices or kidney mince along with citrate or cis-aconitate, no excess isotope was found in the citrate at the end of the incubation period. Acetate was therefore not in equilibrium with the added citrate. However if heavy-carbon-containing oxalacetate and acetoacetate were added to the preparations, the citrate formed had a heavy carbon excess in the primary carboxyl carbon. This would seem to establish that acetoacetate, at least, is oxidized by way of a cycle involving citric acid.

From these reports acetate seems to be oxidized via the

Krebs cycle in kidney preparations and liver slices and although acetate alone did not form acetoacetate in the liver enzyme preparations of Lehninger and Crandall, when pyruvate and acetate were incubated with these preparations part of the acetoacetate formed came from the acetate.

Fatty acids are formed from ingested acetate. Rittenberg and Bloch (72) found that fatty acids formed in rats after ingestion of acetate containing both heavy carbon and deuterium, were found to contain both of these isotopes. The fatty acids thus formed had a carboxyl heavy carbon concentration twice that of the entire chain, which suggested that the fatty acid chain had every second carbon atom isotopic, and that it was therefore formed by condensations of the acetate, or two-carbon units formed from acetate, containing the heavy carbon labelled carboxyl group. Bloch and Rittenberg (7) also found that the cholesterol isolated from rats, after ingestion of deuterium labelled acetate, contained appreciable amounts of the isotope, and that only acetate, or substances from which acetate may be formed in the body, would take part in the metabolic synthesis of this substance.

Later the same authors, (8) using acetate labelled with heavy carbon in the carboxyl group and with a deuterium tagged methyl group, found that about one half of the cholesterol carbon came from the ingested acetate, and that both carbons of the acetate, which were in both the ring structure and side-chain of the steroid, were used in the metabolic synthesis of cholesterol.

It is interesting to note that previous to the isotope experiments on liver and kidney preparations described above,

Elliott and his coworkers had found that rabbit (17) and rat kidney cortex slices (18) and rat liver slices (19) could utilize significant amounts of added acetate under aerobic conditions, while in rat testis (19), acetate utilization was found to depend on simultaneous carbohydrate oxidation, and normally proceeded only slowly.

Kleinzeller (31) had found that acetate oxidation by guinea pig kidney cortex slices or minces was completely inhibited by the presence of 0.03 M malonate. This was an indication that the Krebs cycle was involved in acetate oxidation in these preparations.

In a study on isolated perfused rabbit hearts, Barcroft et al (2) found that the beating hearts destroyed a considerable amount of the acetate added to the perfusion fluid. At the end of the experiments, no more residual glycogen was found in a heart which had been perfused with fluid containing acetate, than in one which had had no acetate passing through it. Presumably the acetate had been immediately oxidized and no carbohydrate was stored. Or, alternatively, the acetate was oxidized at the expense of some other metabolite which, of course, may have gone to lipid instead of to carbohydrate stores.

Formation of Acetate From Pyruvate

All animal tissues, and a large number of bacteria have the ability to use pyruvate. In animal tissues, pyruvate metabolism is appreciably faster under aerobic conditions than in anaerobic surroundings, according to Barron and Lyman (4). The main catabolic reactions undergone by pyruvate in animal tissue, dismutation, oxidation, and decarboxylation, all seem

to require cocarboxylase (3).

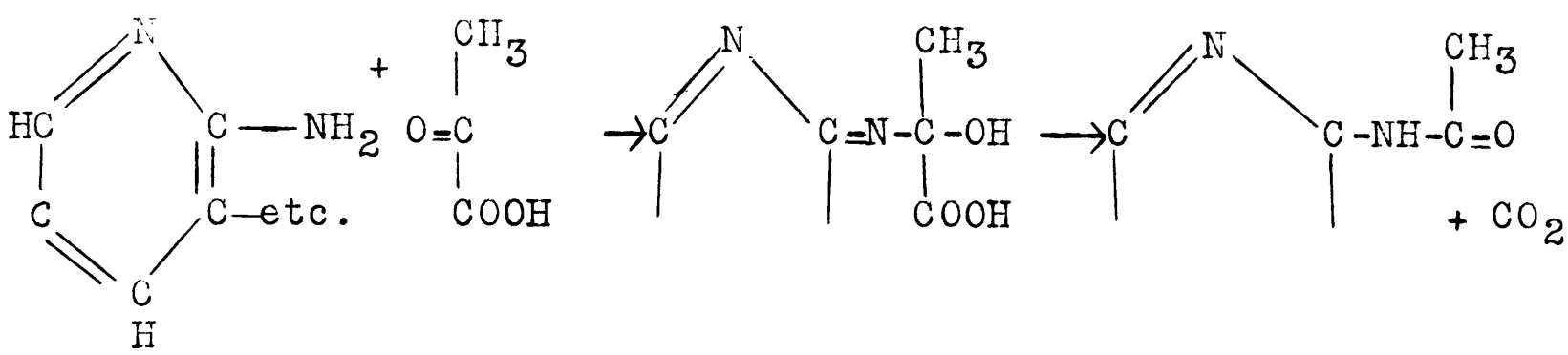
Various authors, principally Krebs et al, and Elliott et al (19,33,37,85), have found that a dismutation of pyruvate to lactate, acetate and carbon dioxide, occurred in a large number of animal tissues. Krebs found that most tissues produced a small quantity of acetate, with testis forming more acetate than most tissues. Elliott et al found very little except in testis. Krebs based his results on an acetic acid determination method which easily gives high results, while Elliott used acid disappearance and carbon dioxide production, determined manometrically, to calculate the amount of acetate formed. Acetic acid determinations with a reliable method, by Elliott et al (20), and by the writer, confirm the small production of acetate by brain tissue. The dismutation reaction described in a previous section, is one of the main pathways for pyruvate metabolism under anaerobic conditions, although some pyruvate is simply reduced to lactate.

Although there have been few studies on the oxidation of pyruvate to acetate and carbon dioxide in animal tissues this reaction has been thoroughly investigated in various bacterial systems by Lipmann (38,39,45), mostly from the point of view of acetyl phosphate formation as an intermediate compound.

A specific pyruvic oxidase has been isolated from pigeon breast muscle by Stumpf et al (79), which does oxidize pyruvate to acetate and carbon dioxide. It requires diphospho-thiamine (cocarboxylase) as the only addition, and is not inhibited by fluoroacetate, fluoride, malonate, or iodoacetate. Arsenite, however inhibits this enzyme by 75% in a concentration which inhibits alpha-ketoglutaric oxidase by only 50%. Pyruvic

oxidase is therefore the more sensitive of the two enzymes to this inhibitor.

Weil-Malherbe (86) has suggested that a hypothetical compound, acetylcocarboxylase, is formed from pyruvate as an intermediate in its oxidation, and that the acetyl group of this compound is the "active acetate", treated in a previous section, which enters into those reactions in which acetate, as such, is inactive. The formation of this compound would go through a Schiff base intermediate which could readily break down to give an available acetyl radical, according to the following series of reactions.



cocarboxylase pyruvate acetylcocarboxylase

(only that portion of the molecule which would take part in the reaction is shown).

Metabolism studies on the malaria parasite by Speck, Moulder, and Evans (76) revealed that acetate is formed from pyruvate by parasitized erythrocytes, considerably more by free parasites, and that this acetate formation is further increased by the presence of high pyruvate concentrations.

The effects of all added substrates and inhibitors were more apparent when added to the free parasite preparations, which were prepared from parasitized erythrocytes by a method

developed by these authors, than when they were merely added to a medium containing washed parasitized erythrocytes.

The addition of small, catalytic, amounts of the dicarboxylic acids of the Krebs cycle decreased acetate formation in free parasites, while malonate increased the formation of acetate in these preparations. It was suggested by these authors that a competition for a two-carbon fragment formed from pyruvate existed in the parasite preparations. The fragment thus formed could either be taken up by the oxidative Krebs cycle or could directly form acetate. The addition of the dicarboxylic acids would have increased the activity of the Krebs cycle and less of the two-carbon fragment would have been able to proceed to acetate. Malonate, which would have blocked the Krebs cycle, would therefore have reversed this state of affairs by reducing the activity of the cycle, and a larger portion of the two-carbon fragment would have appeared as acetate. The competition could, of course, have been for the intact pyruvate molecule instead of for a fragment formed from it. It was concluded, on the basis of this and other evidence, that pyruvate was definitely metabolized via the Krebs cycle in the parasite preparations.

Bartlett and Barron (5), and Kalnitsky and Barron (5a) have found that fluoroacetate inhibits the oxidation of acetate by kidney, liver, and muscle, and by yeast and certain bacteria, (i. e. Corynebacterium creatinovorans), and that in the presence of fluoroacetate, the utilization of added pyruvate is diminished while acetate accumulates.

They concluded that acetate formation by the oxidation of pyruvate is of great importance in animal tissues.

Hutchens and McMahon (28) claimed that in yeast and the Chilomonas paramecium (sic), the extra oxygen uptake produced by the addition of pyruvate to the medium, is inhibited by 50% in the presence of fluoroacetate, and that the extra oxygen uptake produced by the addition of acetate to the medium is inhibited by less than that of pyruvate in the presence of the same concentration of fluoroacetate. Although the figures for the inhibiting fluoroacetate concentrations, as published in the abstract, belie these statements, they are probably a misprint.

The greater inhibition of pyruvate oxidation than of acetate oxidation by fluoroacetate, true for Chilomonas paramecium, is not true for other animal tissues. A release of the inhibition, in yeast only, by the addition of alcohol, has been observed. (Barron and Black, personal communication to Dr. K. A. C. Elliott).

"In vivo" investigations with fluoroacetate by Chenoweth and St. John (13), have shown that intravenous injections of 0.05-0.10 mg. of fluoroacetate per kilogram of body weight, in dogs, cause the development of seizures in the animals resembling the "petit mal" and "grand mal" attacks of epilepsy. Usually more than the 0.10 mg. per kilogram dose was needed to produce the "grand mal" type of attack in the animals. Evidently interference with metabolism of the acetyl group affects the functional activity of nervous tissue.

Formation of Acetate in the Liver of Intact Animals

Weinhouse et al (88) found that considerable amounts of acetate were formed in liver slices incubated with isotopic acetate. The acetate formed by the liver during the experimental period diluted the isotopic acetate carbon which appeared in the ketone bodies formed by the liver by about 50%.

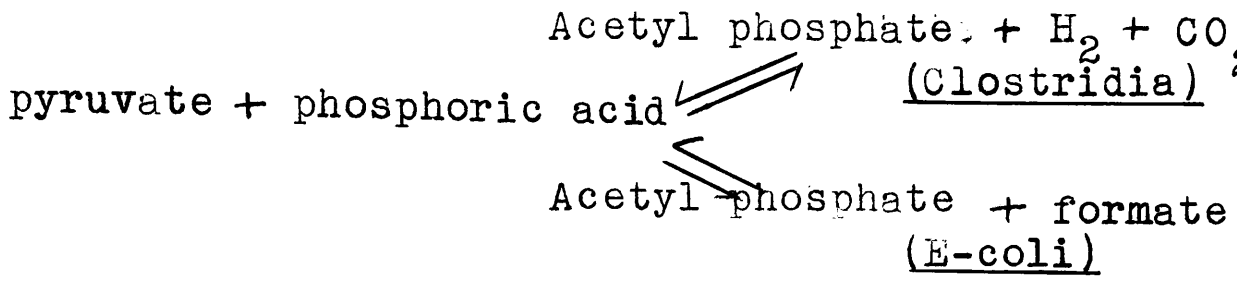
Rittenberg and Bloch (8) in the course of sulfanilamide acetylation studies on the intact rat, found that the dilution of deuterium tagged acetyl groups during the formation of acetylsulfanilamide from ingested acetate, containing deuterium, corresponded to an acetate production of approximately 15-20 mM (9-12 mg.) of acetic acid per hundred grams of rat tissue per day.

Acetyl Phosphate in Bacterial Metabolism

Acetyl phosphate, which is the anhydride of phosphoric and acetic acids, contains the "high-energy phosphate bond" which plays such an important part in present-day metabolic schemes. This concept of "high-energy" bonds was originally proposed by Lipmann and has been elaborated by him (45) and by many other workers. Lipmann has suggested that acetyl phosphate may well be the elusive "active acetate."

In bacterial metabolism various workers have found that the Clostridia produce acetate, hydrogen, and carbon dioxide from pyruvate, while Escherischia coli forms acetate and formate from the same substrate. After the isolation of acetyl phosphate as the actual reaction product in Clostridium butylicum by Koepsell et al (32) and E-coli by Utter et al (82), the reaction, which had previously been termed hydroclastic, was renamed phosphoroclastic by Lipmann. The reactions are

believed to take place according to the following mechanism.



Acetyl phosphate has also been found to be a product of pyruvate metabolism in Bacillus acidificans longissimus by Lipmann (40).

In isotope studies on cell free extracts of Cl. butylicum and E-coli, with heavy carbon in either the formate or acetate added, Utter et al. (81) found that the acetyl phosphate-pyruvate reaction was reversible, as had been suspected from thermodynamic data. The addition of formate containing heavy carbon, along with pyruvate, to the E-coli extracts resulted in the appearance of the isotope in the carboxyl group of the pyruvate isolated at the end of incubation, in large amounts. When acetate containing heavy carbon, and pyruvate were added to the extracts however, little of the isotope was found in the residual pyruvate unless adenosine triphosphate had also been added to the medium. When radioactive inorganic phosphate and acetyl phosphate were added together to an E-coli extract, there was a rapid turnover of inorganic and organic phosphates which agreed well with the formate-pyruvate relationships, and suggested that the limiting factor in the case of the reactions occurring when acetate plus pyruvate were added , was the formation of acetyl phosphate, which would seem to be the more sluggish of the reactions.

Acetyl Phosphate in Animal Tissues

The role of acetyl phosphate in animal tissues is

more obscure than that in bacteria, although it has been noted by Long (50) and by Banga et al. (1) that inorganic phosphate and adenylic acid are necessary for pyruvate oxidation in pigeon brain.

Lipmann (42), and Shapiro and Wertheimer (73) have observed that most of the difficulty in studying acetyl phosphate in animal tissues is due to the presence of a remarkably active, probably specific, acyl or acetyl phosphatase, which was found in most of the animal tissues studied, including brain. The enzyme breaks down added synthetic acetyl phosphate so rapidly that little is left after a five minute period of incubation with tissue preparations. The enzyme is inhibited somewhat by the presence of fluoride (42) and to a greater extent by a high concentration of inorganic phosphate (73, 82), by hexose phosphate, pyrophosphate, and, less actively, by sulfate, oxalate, and by citrate (42). It is also inhibited by some substances of high molecular weight such as the nucleic acids and hyaluronic acid (45). The action of this enzyme probably explains the negative findings obtained by Ochoa et al. (66) in attempts to prove a transfer of high energy phosphate from acetyl phosphate to adenylic acid.

By converting enzymically formed acetyl phosphate into acetohydroxamic acid as soon as it is formed, by the reaction with hydroxylamine described in a later section, the enzymic destruction of the acyl phosphate is prevented. Using this technique, Lipmann and Tuttle (43) found that the transfer of phosphate from added adenosine triphosphate

to added acetate, which occurred even with low (0.001 M) acetate concentrations, was quite an active process in pigeon liver extracts. This "trapping" method cannot, however, be used in studying tissue metabolism due to the formation of oximes of carbonyl containing compounds by hydroxylamine, a reaction which proceeds easily even at room temperature and which would interfere with the metabolism of the tissue at several points.

It has been suggested by Shapiro and Wertheimer (73) that when acetyl phosphate is formed as an intermediary metabolite, it is probably kept in the necessary concentration by the inhibition of acetyl phosphatase by the cellular phosphate concentration, which they evidentally assumed to be high enough for this purpose.

Acetylation of Sulfanilamide and Amines

Another aspect of acetate or "active acetate" metabolism, is the mechanism of acetylation of various substances including sulfanilamide, foreign amines and choline.

The acetylation of sulfanilamide in the human was discovered in 1937, by Marshall et al. (57) and by Fuller (25). The reaction is not, however, a general one in the animal kingdom, for it is not exhibited by dogs, birds and cold-blooded animals (58) and although Stewart et al. (78) found that the liver was the site of sulfanilamide acetylation, van Winkle and Cutting (83) found that other organs could take part in the process in the cat.

Acetylation of sulfanilamide in rabbit and human liver

slices is limited by the rate of acetate formation by the by the tissues. Added acetate, or substances from which acetate may be produced in the tissues, increase the rate of acetylation. Anaerobic conditions, or the presence of inhibitors such as arsenious oxide or iodoacetamide decrease acetylation, which is mainly due to a decrease in acetate formation, according to Klein and Harris (30). Formation of acetyl-sulfanilamide is blocked in the intact rat by the administration of glucuronic acid, although the investigators, Martin et al. (59), could find no evidence of the formation of the glucuronide of sulfanilamide.

Rittenberg and Bloch (8) claim that acetic acid is an effective, and in fact the only, acetylating agent for aromatic amines, sulfanilamide and p-aminobenzoic acid. Phenylaminobutyric acid, however, can also be acetylated by a mechanism involving pyruvate, since the feeding of deuterium labelled alanine to rats along with phenylaminobutyric acid, resulted in the excretion of the acetylated derivitive of the amine containing deuterium in its acetyl group.

In strong (0.4 gm. of tissue per ml.) isotonic pigeon liver homogenates, Lipmann (42) was able to demonstrate that acetylation of sulfanilamide went on without the addition of metabolites, but that the rate of acetylation was doubled on the addition of acetate to the medium. Pyruvate and aceto-acetate were only about one half as active as acetate itself, and acetoin (16, 42) caused only a small increase in the acetylation rate. Under anaerobic conditions, the addition of adenosine triphosphate to the homogenate increased anaerobic

acetylation to near the rate found for aerobic conditions. It was concluded that the addition of adenosine triphosphate replaced respiration as a supply of high energy in these preparations. Acetyl phosphate as such, was found to be inactive as an acetyl group donor. The effect obtained on the addition of this compound did not exceed that of free acetate alone.

Fishman and Cohn (24) have concluded that the actual acetylating substance should readily exchange deuterium with body water which has been enriched with this isotope in the form of deuterium oxide. In the experiments done by these authors, the excreted acetyl derivatives of sulfanilamide and p-aminobenzoic acid were found to contain deuterium in the acetyl group when deuterium oxide (D_2O) was administered along with these compounds. The fact that pyruvate and acetate, when allowed to stand in water containing deuterium oxide, showed little or no exchange reactions with the deuterium of the medium, seems to point to the intermediate formation of "active acetate" as the direct acetylating compound.

Bloch and Rittenberg (7) found that substances which yield "active acetate" in the tissues, such as ethanol, butyric acid, alanine, and valeric and myristic acids, when labelled with deuterium and fed to animals along with phenylaminobutyric acid, all yield an acetylated derivative of the amine which contains deuterium in the acetyl group.

The acetylating system of brain and liver has been partially purified by Kaplan and Lipmann (29). This enzyme suspension accepts only acetate as an acetyl group donor.

The coenzyme for acetylation, common to the acetyl-

ation systems of both brain and liver tissue, has been recently found by Lipmann et al. (46) to be a pantothenic acid derivitive.

Acetylation of Choline

One of the phases of the functional activity of nervous tissue is the synthesis and hydrolysis of acetylcholine, the effector substance of nervous activity. Neither the various complex theories of the function of acetylcholine, nor the highly developed hydrolytic mechanism of its destruction by the enzyme cholinesterase will be enlarged upon here, but only those phases of the acetylcholine system which involve acetate, or the acetylating substance, will be treated.

Mann, Tennenbaum and Quastel (55) showed that pyruvate, lactate, and glucose all stimulate acetylcholine synthesis in brain minces, but they found that acetate itself had little or no effect on the synthesis. No acetylcholine was found to be synthesized in the absence of pyruvate or glucose in aerobic suspensions and slices (54). With high potassium concentrations (0.03 M.),polyneuritic pigeon brain slices synthesized less acetylcholine than slices from normal brains, but the normal rate occurred on addition of thiamine to the avitaminotic tissue. With potassium concentrations approximating those of blood serum, the synthesis of acetylcholine is low, and is the same in both polyneuritic and normal pigeon brains (56).

In 1943, Nachmansohn and Machado (61) isolated an enzyme system from brain tissue which was capable of anaerobically synthesizing acetylcholine from choline and an acetate donor, in he presence of potassium ions, adenosine triphosphate and fluoride. The synthesis was found to be stimulated by the presence

of citrate, cysteine and glutamate. Calcium ions and keto-acids inhibit the synthesis of acetylcholine by this enzyme system, while potassium ions accelerate it (62). The fluoride was added to the system to inhibit adenosine triphosphatase. The choline acetylating enzyme is one of the group of enzymes which contain sulfhydryl groups, which are readily oxidatively inactivated, but which may be restored to activity by the addition of cysteine. Citrate is probably on acetyl donor in this system (see below) but the role of glutamate is obscure.

A coenzyme which may be obtained from boiled yeast extract, or from a brain extract, was found to be necessary for the activity of this enzyme system. As was previously noted, this coenzyme was found to be identical with the coenzyme necessary for sulfanilamide acetylation in liver (44) and which was found to be a pantothenic acid derivitive (46).

Nachmansohn et al. (61) have found that the enzyme, choline acetylase is limited to nervous tissue, the nerve axon (63) (from which it has entirely disappeared seventy-two hours after section of the nerve) and to cardiac muscle (64).

Lipton and Barron (47), in experiments on the anaerobic synthesis of acetylcholine by brain enzyme extracts, claim that donors of "active acetate" to the system, include acetoacetate, citrate and cis-aconitate. These authors propose the following reactions as the source of the acetyl group in acetylcholine synthesis in the presence of the enzyme system.

1. acetoacetate + H_2O $\longrightarrow$ 2 "active" acetate

2. citrate $\longrightarrow$ oxalacetate + "active" acetate

and 3. "active" acetate + choline $\longrightarrow$ acetylcholine

Pyruvate was considered to be the immediate source of acetyl groups under aerobic conditions, but the actual donor was considered to be an "active" acetate produced by some group of reactions as are outlined above. The synthesis of acetyl choline was considered to be a two-step reaction with (1) the formation of the "active" acetate and (2) acetylation of choline with the "active" acetate thus formed. Whether the acetylation coenzyme and adenosine triphosphate are necessary for the first, or the second reaction, is not known. In this connection, the explanation given by Lipmann (42) for the absence of an acetyl phosphate effect on the rate of sulfanilamide acetylation in liver homogenates, may be mentioned. Lipmann considers that acetate and sulfanilamide are "enzymically induced to form a complex with adenyl pyrophosphate, which exergonically breaks up into acetylsulfanilamide, adenylic acid and inorganic phosphate." Some such mechanism may well be applicable to the synthesis of acetylcholine.

It has been observed by many authors (22, 23, 52, 61) that acetate itself is not available for acetylcholine synthesis either aerobically or anaerobically. However, Kaplan and Lipmann (29) claim that a purified acetylating system has been prepared by them from brain and liver tissue which accepts only acetate as an acetyl donor. Citrate was not active with their preparations. These observations are contrary to those of Lipton and Barron (above).

From all this work it is evident that "active" acetate, which may be formed from other substances, or perhaps from acetate itself, plays an integral part in acetylcholine

synthesis and it is therefore a major factor in the functional activity of nervous tissue.

EXPERIMENTAL

All of the experimental work to follow was done on"homogenates", that is suspensions, of whole rat brain.

The respiration of isotonic suspensions of brain tissue and brain slices has been described and compared by Elliott and Libet (21). Homogenization of tissue eliminates the tedious work of slicing, draining, weighing, etc., necessary when using sliced tissue, and enables more accurately comparable samples of tissue to be taken. Duplicate oxygen uptake figures obtained with brain slices do not agree very closely and the respiration rate of different areas of the same brain varies (21). Slices cannot be drained thoroughly without damaging the tissue, and therefore moist weights of the slices cannot be as accurately determined as might be desired,* while determination of final dry weights is inaccurate due to some disintegration of the slices occurring during the experimental period, unless special methods, such as that of Stadie (77) are used. In Stadie's method the medium is treated with trichloracetic acid and the slice and precipitate are collected in a Gooch crucible for drying and weighing.

When homogenates are used, the time taken from the death of the animal to the beginning of the experimental period is much shorter than that needed for slice preparation, and the loss of activity which may occur during the longer

* While this work was in progress, a method for preparing slices without moistening and so obtaining correct fresh weights, was perfected in this laboratory.

period needed for slicing and weighing, is avoided.

Duplicate oxygen uptake determinations on samples of the same homogenate varied by only about 2%, although there was a maximum variation of about 30% with brain homogenates from different animals. Similar R. Q. values were found with both slices and isotonic homogenates. The respiration of suspensions, however, is appreciably less than that of slices. When slices of gray matter were homogenized in isotonic medium, the suspension thus formed respired about 35% less actively than similar unhomogenized slices in the same medium. This difference could be due to the fact that a certain proportion of the structures of the cells necessary for normal metabolism are destroyed by the homogenization process.

Suspensions prepared in hypotonic media (distilled water or hypotonic saline or phosphate) respire only about one fourth as actively as isotonic suspensions, and although these preparations are more useful for studying the effects of added catalysts or other substances, the disruption and cytolysis of the cells make for more unphysiological conditions than are encountered when an isotonic homogenization medium is used. Reiner (68), has shown that water homogenates of brain tissue will respire even faster than slices if a number of catalysts and intermediary metabolites are added to the medium. Several authors (e. g. Racker and Krimsky, (69) Meyerhof, (60)) have shown the same for glycolysis by brain homogenates and extracts.

Procedure

The rats, a pure strain of hooded rats, were decapitated with shears and the whole brain dissected out. Major membranes were removed with fine forceps and the brain wiped free of blood

with small pieces of filter paper. The brain, or the pooled brains of several rats, was transferred to a previously weighed Potter-Elvehjem tube (68) and the total fresh weight of the tissue was determined.

Enough Ca-free Ringer-0.017 M phosphate, or other medium, was then added to give the required tissue weight per ml. of suspension, usually 150 mg. per ml. One gram of fresh whole rat brain was considered to contribute 1 ml. to the total volume of the suspension and that amount was subtracted from the volume of medium added.

When insulinized brain tissue was desired, several rats were injected intramuscularly with enough insulin solution, (6.5 units per rat over a period of about two hours) to cause them to become comatose or have hypoglycemic convulsions. When the animals were completely comatose they were decapitated and the rest of the procedure described above was carried out.

The entire operation, from the decapitation of the animal to the beginning of the experimental period seldom took more than thirty minutes.

Samples of the suspension, usually 3 ml., were pipetted into vessels of Barcroft differential manometers. If the respiration of the tissue was to be followed, alkali and rolls of filter-paper were placed in the center well of the vessels and the respiration of the tissue was followed manometrically in the normal fashion, after an equilibration period of fifteen minutes at 38°. At the end of the incubation period, after the removal of the alkali papers, if they had been used, the samples of

homogenate from two vessels were pooled and an acetic acid or an acetyl phosphate determination was carried out on a sample of the pooled homogenate,

ANALYTICAL METHODS

Acetic Acid

Distillation methods for the determination of acetic acid, in the presence of pyruvic and lactic acids, have been described by Weil-Malherbe (84), by Krebs and Johnson (53), and others.

In the method of Weil-Malherbe, the tissue preparation after centrifugation, is treated with phenylhydrazine in the cold to remove pyruvate and the protein left in solution. The solution is then filtered and a sample distilled in the Parnas apparatus at a pressure of 12 mm. of Hg. and the distillate titrated with standard CO_2-free NaOH. Recovery of acetate added to tissue preparations, is 80% with this method.

Krebs and Johnson remove pyruvate and protein by treatment of the tissue with the Salkowski-Van Slyke copper-lime precipitation method, and steam distil the filtrate, collecting the distillate in standard NaOH and then back-titrating.

A simpler and reliable method for the determination of acetate in tissue preparations is described by Elliott et al (20). In this method, protein is removed from test solutions by $Zn(OH)_2$ precipitation and a portion of the clear solution thus obtained is distilled and then redistilled after evaporation of the first distillate to a small volume. The distillations are done at atmospheric pressure which eliminates the cumbersome apparatus necessary for distillations at a low pressure.

When solutions containing lactate, pyruvate and chloride are acidified and steam distilled, lactic and pyruvic acids, particularly pyruvic, distil over appreciably, and if the pH of the solution is too low, HCl may also distil over. By acidifying with KH_2PO_4 (pH4.4-4.7) for a first distillation, acetic acid distilled over without appreciable amounts of HCl also coming over. Although pyruvic and lactic acids do, to some extent, distil with steam, distillation and redistillation completely eliminates any appreciable interference of these substances in the determination of acetic acid. This method is not, of course, specific for acetic acid, but it is unlikely that any other steam volatile acid could be produced in these studies in amounts capable of affecting the titrations.

The distillation apparatus used at first was a set of flasks with ground glass joints, copied from a set used by Dr. W. C. Stadie. These flasks had evidently not been exactly copied, for they were found to have rather narrow necks, which interfered with proper distillation. This error brought out an important point in the design of the apparatus.

It was found that fluid collected at the constriction in the neck of the flask during steam distillations and prevented free passage of steam to the condenser. Droplets of this fluid were apparently constantly being blown into the Kjeldahl trap situated between the flask proper and the condenser.

The foaming which occurred during the second distillation, in which syrupy phosphoric acid was used as the acidifying agent, contaminated this reservoir of fluid in the neck of the flask and the droplets, when blown over, contained enough phosphate to

affect the titration of the distillate.

The large opening at the end of the steam inlet tube tended to cause splashing of the distilling fluid, due to the large bubbles which emerged from it. As a result of these conditions large and variable blank titrations were obtained.

On consulting Dr. K. A. C. Elliott, it was decided to have completely new flasks made, which would embody features designed to eradicate the error sources described above. The new flasks (Fig. 1) had necks((A) with twice as much clearance as the previous ones, so that no fluid collected in the necks during distillation, and widened outlet tubes leading to the Kjeldahl traps (B). A perforated bulb (C) was substituted for the single opening on the ends of the steam inlet tubes which enabled even, fine bubbling of steam through the distilling fluid to be maintained. These modified flasks almost completely eliminated accidental phosphate contamination of the distillates and enabled distillations to carried on at a much faster rate than was before possible. These modified flasks were used for all of the experiments on acetic acid production reported.

Distillation of solutions containing 2 mg. each of lactic and pyruvic acids resulted in a small blank titration value (0.10 to 0.13 ml. of 0.008 N NaOH) which was little higher than the blank obtained from the distillation of water alone (0.10 ml. of 0.008 N NaOH).

Figure 1

Acetic Acid Distillation Flask

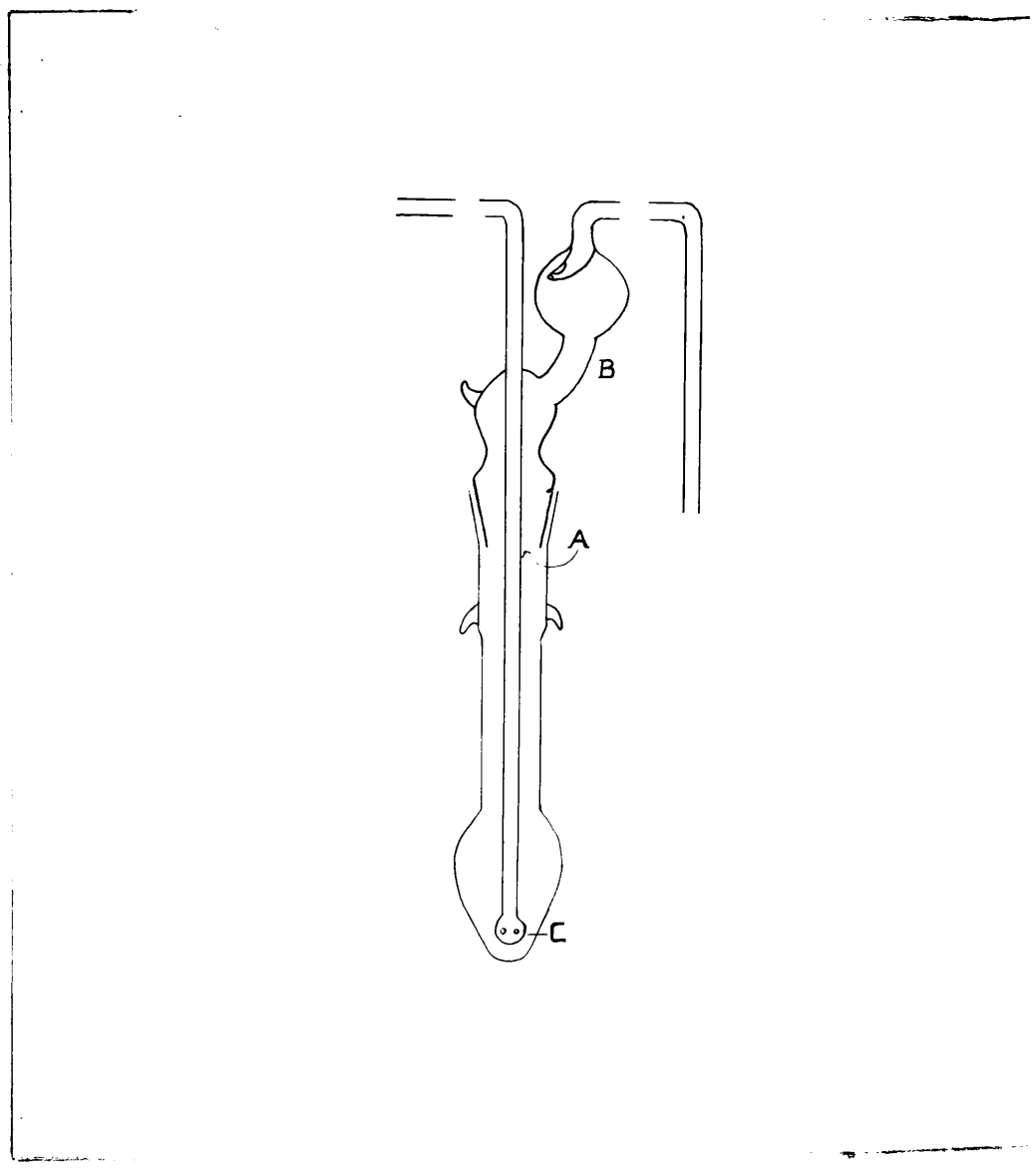


Diagram of the distillation apparatus with the modifications described in the text. The clearance at the neck of the flask, between the steam inlet tube and the inner joint of the neck is 5 mm.

The acetic acid determination method of Elliott et al. and as used by the writer for this work, is here given in detail.

In order to prepare a protein-free solution for acetate determinations, 5 ml. of the brain tissue suspension were added to a graduated 15 ml. centrifuge tube containing 3.5 ml. of 10% $ZnSO_4.7H_2O$ solution. The mixture was stirred thoroughly and 3.5 ml. of 0.5 N NaOH were added slowly with constant stirring, and enough distilled water was added to bring the volume to 15 ml. The tubes were spun in a centrifuge at 2500-3000 R. P. M. for five minutes, and two 5 ml. samples of the clear supernatant fluid were taken for duplicate acetic acid determinations. The samples were added to the steam distillation flasks described above, and 2 gm. of KH_2PO_4 were added, the acidified solution was then distilled. When 50 ml. of distillate had collected, it was made alkaline with a few drops of 0.6 N NaOH to the end point of brom-thymol blue and was then evaporated to 2-3 ml. on a hot plate with small glass beads added to prevent spattering. The fluid was then reintroduced into a distilling flask which had been thoroughly rinsed and steamed in the meantime, and 0.5 ml. of syrupy phosphoric acid was added. The mixture was steam distilled as before and 35 ml. of distillate collected in a round-bottomed 50 ml. centrifuge tube. The volume of fluid in the distillation flasks was kept at a constant 5 ml. by adjusting the height of electric heating coils below the flasks.

Air, freed from CO_2 by passage through a soda-lime tower, was bubbled through the second distillate for 6-10 minutes, and the distillate was then titrated with freshly standardized, CO_2-free, 0.005-0.008 N NaOH, mixing being achieved by a continuous stream of washed air bubbles.

Recoveries of acetic acid in the presence of pyruvic and lactic acids are reported in Table I.

TABLE I

Recovery of Acetic Acid in the Presence of Pyruvate and Lactate

Five ml. of aqueous or Ringer solution containing the amounts of acetic acid shown and 2 mg. each of pyruvate and lactate were used in each experiment.

Mg. acetic acid added	Mg. acetic acid recovered	% recovery
0.269	0.272	101
0.354	0.343	97
0.474	0.459	97
0.474	0.483	102
0.474	0.426	94
0.474	0.485	102
0.474	0.474	100
0.708	0.683	97

Acetyl Phosphate

The colorimetic method for the determination of acyl phosphates, as published by Lipmann and Tuttle (41) was tried on brain tissue suspensions.

The basis of the acyl phosphate method is the reaction between an acyl phosphate and hydroxylamine:

$$R\text{-}CO\text{-}OPO(OH)_2 + NH_2OH \dashrightarrow R\text{-}CO\text{-}NHOH + H_3PO_4$$

(acyl phosphate) (hydroxylamine)(hydroxamic acid)

The product of this reaction, the corresponding hydroxamic acid, gives a color with ferric chloride in acid solution, which

may be measured colorimetrically as follows:

A solution of 2M hydroxylamine·HCl, pH 6.4, is freshly prepared by mixing a volume of 4M hydroxylamine·HCl with an equal volume of 3.5 N NaOH. A volume of test solution (0.5 to 1.0 ml) is added to a mixture of 1 ml. of the 2M hydroxylamine. HCl and 1 ml. of 0.1 M acetate buffer, pH 5.5. The volume of this mixture is adjusted to 3 ml. and the mixture is allowed to incubate for ten minutes at room temperature. Then 1 ml. each of hydrochloric acid, (diluted 1 part concentrated acid to 3 parts water) 12% trichloracetic acid, and 5% ferric chloride solution in 0.1 N HCl, are added in that order. The protein precipitate is either thrown down in the centrifuge or filtered off, and a portion of the remaining clear solution (4 or 5 ml. are taken, depending on the amount of protein precipitate) is read in the Klett-Summerson or Evelyn colorimeter with a 540 filter. The reading should be taken 5 to 30 minutes after the addition of the ferric chloride solution. The color developed by this procedure ranges from a brownish-purple to a dark purple, depending on the concentration of the ferric-hydroxamic acid complex.

Standard solutions are prepared from succinic anhydride, which is converted to its monohydroxamic acid by the action of a concentrated solution of hydroxylamine·HCl, and diluted to any required concentration for use in standard and recovery determinations. The color developed by the standard is consistently 80% of that formed from an equivalent amount of acetohydroxamic acid formed from acetyl phosphate. A standard curve for recovery determinations is made.

This method, as described above, was found to be completely unsatisfactory for use on brain suspensions.

Protein precipitation with trichloracetic acid in a final concentration of 2%, as outlined in this method, worked all right with the tissue preparations and reagents in the absence of hydroxylamine, but when that substance was present in the 0.3 M concentration and pH of 6.4 required by the method, trichloracetic acid invariably failed to give clear solutions after centrifugation.

The alternate protein precipitation procedure, suggested by Lipmann and Tuttle for use in the presence of a high phosphate concentration, which depresses the color developed by 13% for every 100 micromoles of phosphate present in the sample, was also attempted. This method evidently had not been as fully tested experimentally as it should have been, for if this method were followed exactly as it was published, it failed to give clear protein-free solutions, nor did it eliminate the depression of color caused by high phosphate concentrations.

In this alternate procedure 2 ml. of the test solution are added to a mixture of 2 ml. of the 2M hydroxylamine and 2 ml. of the acetate buffer and the resulting mixture is allowed to stand at room temperature for ten minutes, as before. At this point 1 ml. of a mixed solution of 3.4% $ZnCl_2$ and 5.6% $CaCl_2$ is added, and 1 ml. of 0.3N NaOH is added to complete protein precipitation. The mixture is then centrifuged and a portion of the supernatant fluid taken for colorimetric reading.

When the amounts and concentrations, suggested above, were used with brain tissue homogenates, it was found to be impossible to obtain clear supernatant fluids when 2M hydroxylamine was present. The buffering action of this reagent probably

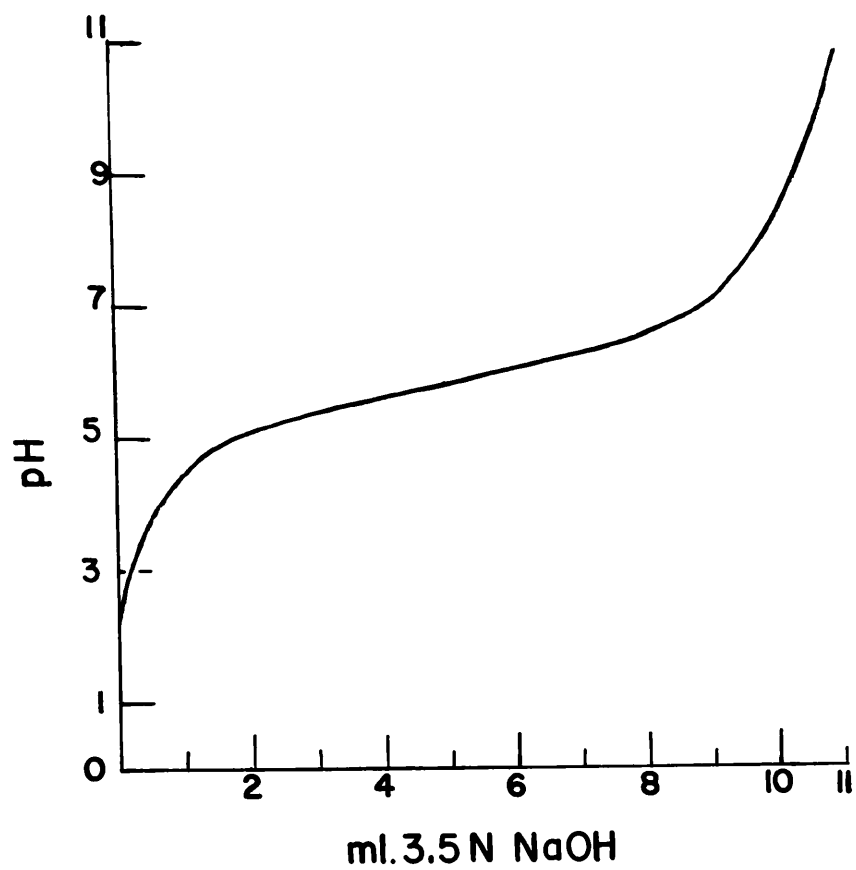
prevented complete protein precipitation by keeping the pH of the solution too low for $Zn(OH)_2$ to come down, and cloudy solutions were always obtained which were entirely unsuitable for colorimetric determinations.

Complete protein precipitation was found to be very difficult to obtain in the presence of strong hydroxylamine. With trichloracetic acid in final concentrations ranging from 3% to 12%, no clear solutions could be obtained from homogenates, although any of the precipitant concentrations in this range would do so in the absence of hydroxylamine. Perchloric acid also failed as an efficient precipitant under these conditions. Metaphosphoric acid gave clear supernatants even in the presence of hydroxylamine, but was found to be unsuitable for use with this method for it prevented the development of a color with ferric chloride. Presumably metaphosphoric acid forms a complex with the ferric ion and prevents the latter from reacting with the hydroxamic acid to produce the color of the ferric-hydroxamic acid complex, on which the method depends.

In Fig. 2 a titration curve is shown which demonstarates the strong buffering action of the hydroxylamine solution. The addition of 1 ml. of 0.1M acetate buffer in the method of Lipmann and Tuttle is quite pointless, since the buffering effect of this amount of acetate is negligible compared to that of the hydroxylamine hydrochloride. This addition of acetate was therefore omitted in the method finally adopted.

FIGURE 2

Titration of Hydroxylamine-hydrochloride with Strong Alkali



Eleven ml. of 3.5 N NaOH were added to 10 ml. of 4 M hydroxylamine HCl solution, and the pH measured with a Beckman pH meter after the addition of each ml. of alkali.

The modification of the suggested $Zn(OH)_2$ precipitation method which was worked out and which gave satisfactory results even in the presence of hydroxylamine is described below:

Two ml. of suspension were added to 2 ml. of 2M hydroxylamine, made up to have a pH of 6.5-6.6, in a graduated 15 ml. centrifuge tube and the mixture was allowed to stand for 10 minutes at room temperature with occasional stirring. One ml. of 0.6 N NaOH was then added, which brought the pH of the mixture to about pH 7. Then 2 ml. of the $ZnCl_2$-$CaCl_2$ solution were added with stirring, and finally 2 ml. more

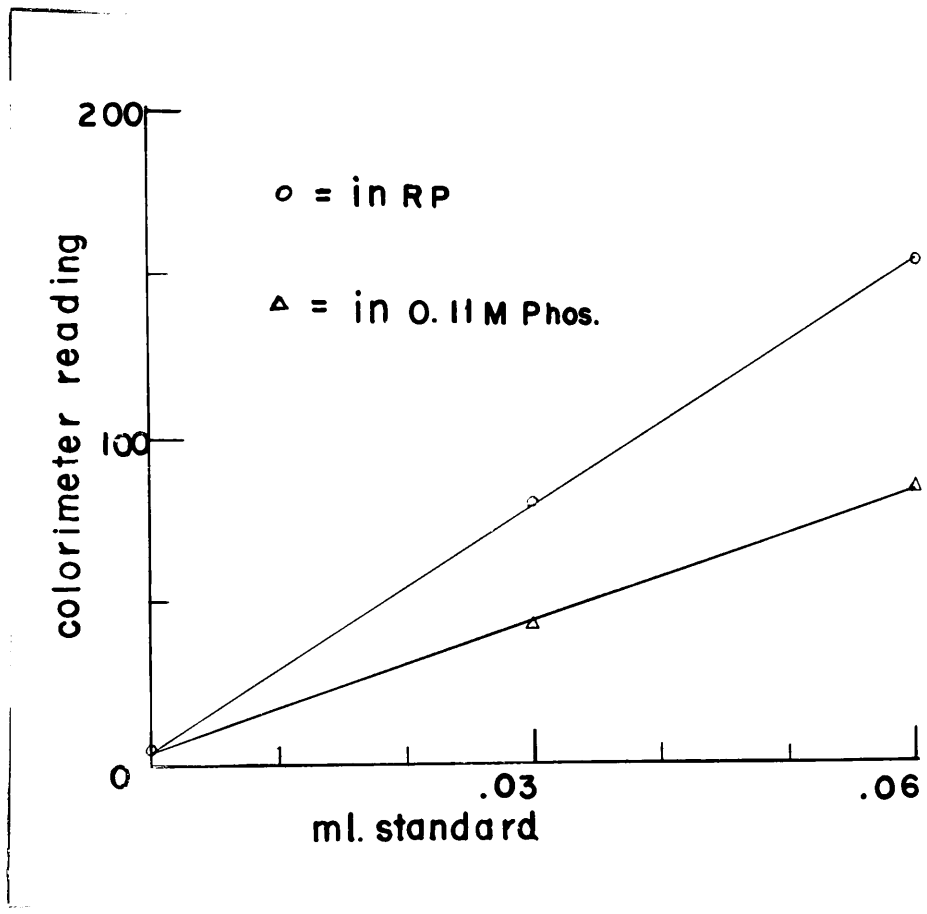
of 0.6 N NaOH were added slowly with thorough mixing. The tubes were then spun in a centrifuge at 2500 R. P. M. for 5 minutes and 3 ml. of the clear supernatant fluid were taken for colorimetric reading. One ml. each of the hydrochloric acid solution and the ferric chloride solution were added, and the resulting solutions were allowed to stand for five or more minutes before reading in the Klett-Summerson or Evelyn colorimeter with a 540 filter.

Higher readings were always obtained from those standard tubes in which the standard solution had been added to Ringer-phosphate, than from those in which 0.11 M phosphate buffer had been the medium, (Fig. 3). This discrepancy was found even though the amount of Ca ions added in the 2 ml. of $ZnCl_2$-$CaCl_2$ solution was in excess of the amount needed to completely precipitate all of the phosphate in the solution as its calcium salts.

Recoveries of succinhydroxamic acid standard solutions added to brain tissue suspensions were good (94 to 106%), from both suspensions made in Ringer-phosphate and in 0.11 M phosphate buffer.

FIGURE 3

Succinhydroxamic Acid Standard Curves in Ringer-phosphate and 0.11 M Phosphate.



Succinhydroxamic acid standard was added to 2 ml. of either Ca-free-Ringer-0.017 M phosphate, or to 0.11 M phosphate buffer, in the quantities indicated, and the modified acyl phosphate determination method described in the text was carried out on this solution. The colorimetric determinations were done with the Klett-Summerson colorimeter using a 540 filter.

RESULTS

Acetic Acid

Since pyruvic acid is known to give rise to the two carbon fragment, "active acetate", in animal tissues and to be oxidized to acetate itself in various bacterial systems, it was decided to attempt to find the extent to which acetate may be formed from pyruvate and from glucose, which can give rise to pyruvate, in the course of the metabolism of brain tissue suspensions.

Long (48), in experiments on aerobically incubated pigeon brain minces with added pyruvate, and using the distillation method of Weil-Malherbe (84), found that 26 micromoles of acetate, per gram of tissue, were formed by his preparation in a 3 hour experimental period.

However, Elliott et al. (20) using a more reliable estimation method, found that only small amounts of acetate were produced aerobically by isotonic rat brain suspensions. Incubation of tissue suspensions in the presence of pyruvate, glucose, or with glucose plus pyruvate, by these authors, produced, respectively, only 9.9, 4.3 and 8.8, micromoles of acetate per gram of tissue, in a 2 hour incubation period.

The writer, using isotonic rat brain suspensions prepared in the same manner as those of Elliott et al. and using the modified form of their distillation apparatus described above, has found that an average of 6.6 micromoles of acetate were formed from pyruvate, and 2.5 micromoles from glucose in a 1 hour incubation period, which are in approximate agreement with the values for acetate production found by Elliott et al. in a 2 hour

incubation period. When their tissue preparations were shaken for 2 hours with no added substrate, Elliott et al. found that only 5.3 micromoles of acetate per gram of tissue were produced, this amount was slightly lower (2.2 micromoles per gram) when suspensions prepared from the brains of rats rendered comatose by insulin injections were used. These observations were confirmed by the writer, who found that 3.2 and 1.3 micromoles of acetate were formed by suspensions of the brains of normal and insulinized rats, respectively, in 1 hour's incubation with no added substrate.

In Table II the results obtained by the writer on incubation of isotonic tissue suspensions, with pyruvate, glucose and with tissue from normal and insulinized animals, are presented.

There is some lactate present in brain tissue even without added glucose. Elliott et al. (20), showed that this amount was enough to support nearly full respiration for about an hour. This lactate, if oxidized via pyruvate, would maintain, for a time, nearly as high a pyruvate concentration as would be encountered with added glucose, and so the amount of acetate found with no addition would be expected to be about the same amount as found with added glucose.

Elliott et al. (20) also showed that the oxygen uptake and R. Q. values of tissue from insulinized rats, when incubated with no added substrate, were lower than the same values found for tissue taken from normal uninsulinized animals. The lowering of acetate formation by insulinized tissue,

TABLE II

Acetic Acid Formed in Isotonic Rat Brain Suspensions in the Presence of Pyruvate, Glucose and With No Addition

Three ml. of isotonic, Ca-free Ringer-0.017 M phosphate suspension were shaken for one hour in Barcroft vessels at 38°. The added substance, if any, was added to the vessel before the introduction of the suspension.

Acetic Acid micromoles/gram of Tissue		
Initial	Found After Incubation	Formed*
	0.025 M sodium pyruvate	
-	10.6	-
0.6	7.5	6.9
0.5	6.3	5.8
0.5	9.7	9.2
3.9	8.7	4.8
1.8	5.4	3.6
0.5,0.7,2.2,3.2,2.0	6.8	4.6
average 1.6	average 7.8	average 6.2
	0.01 M glucose	
	5.3,2.2	
	6.3,5.0	
		average 3.2*
	no addition	
	3.8,6.2	
	6.2,5.0	
		average 3.2*
	no addition (insulinized rats)	
0.6	1.9	1.3

*The average initial value has been subtracted.

presumably due to the exhaustion of the tissue carbohydrate stores during the hypoglycemic period caused by the insulin injections, is confirmed by the writer in this one experiment.

Acyl Phosphate in Brain Tissue

Although acetyl phosphate has not been isolated from animal tissues, it is conceivable that the breakdown of metabolically formed acetyl phosphate could be the source of the acetate formed when pyruvate or glucose is incubated with brain tissue preparations. Acetyl phosphate would break down in the acidic solutions used in the acetic acid distillation method and the "acetic acid" determined could actually be this compound's breakdown product and not acetate itself.

The modification of the method of Lipmann and Tuttle, described in the section on methods, was used to determine the amount of acyl phosphate present in isotonic rat brain suspensions and to follow the course of acetyl phosphate breakdown when added to these suspensions.

Lipmann (45) found that added synthetic acetyl phosphate was broken down quite rapidly when added to preparations from all tissues including brain. Little of the added acetyl phosphate was left after a five minute incubation period in the presence of tissue, and it had completely disappeared from the preparations after ten minutes.

This rapid breakdown is due to the presence, in all tissues, of the enzyme "acyl phosphatase", mentioned in a previous section, which hydrolyses acyl phosphate to the corresponding aliphatic acid and phosphoric acid.

Lipmann (45), and Shapiro and Wertheimer (73), have shown, however, that the acyl phosphatase may be greatly

inhibited by a high phosphate concentration.

In Fig. 4 the rate of hydrolysis of synthetic lithium acetyl phosphate (kindly supplied by Dr. F. Lipmann) when added to isotonic rat brain homogenates is shown. The acyl phosphate was added to homogenates made up in isotonic Ca-free Ringer-0.017 M phosphate, and to homogenates suspended in 0.11 M phosphate buffer with amounts of KCl and $MgSO_4$ added in amounts equivalent to those in the complete Ringer-phosphate.

In the presence of the Ringer-phosphate tissue suspensions, over two-thirds of the added 0.007 M acetyl phosphate was destroyed in the five minutes incubation period, (Fig. 4). However, when the same amount of acetyl phosphate was incubated with the 0.11 M phosphate tissue suspension, only about one-fifth of it was destroyed during an equal incubation period.

The results of acyl-phosphate determinations done on isotonic rat brain suspensions with pyruvate or glucose added to the suspending medium are shown in Table III.

The amount of acyl phosphate found initially and "pre-initially" in freshly homogenized suspensions varied and was often below the range of the method. The "pre-initial" values given are the amounts found when hydroxylamine was present in the homogenizing medium and thereby trapped any ready-formed acyl phosphate, so that the value obtained should represent the amount of acyl phosphate originally present in the tissue. These "pre-initial" values were occasionally higher than the "initial" value found in the usual way, after homogenization, at the start of the incubation period on a sample of the same brain.

TABLE III

Acyl Phosphate in Isotonic Rat Brain Suspensions

Results are expressed as micromoles of acyl phosphate found per gram of brain tissue, calculated as acetyl phosphate. The suspending medium was Ringer-phosphate unless otherwise stated.

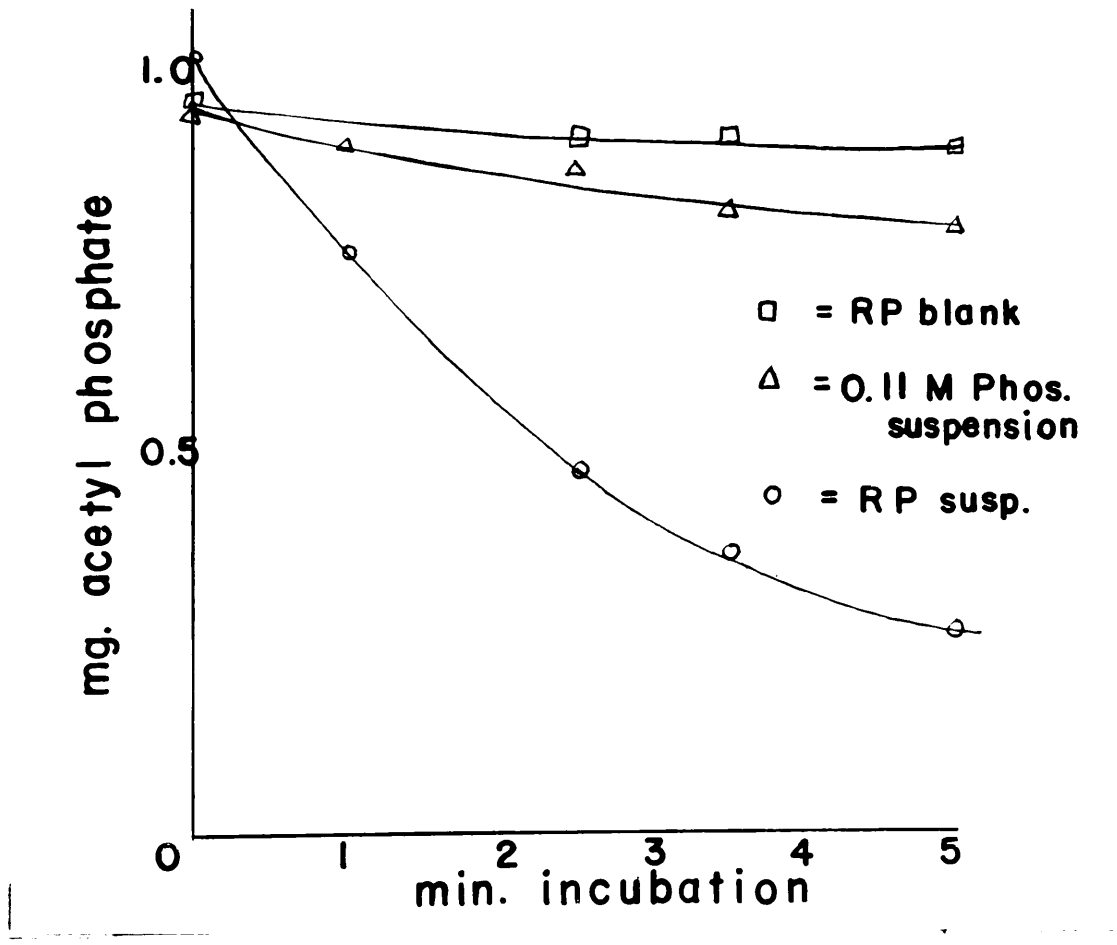
Additions	Micromoles of Acyl Phosphate Found*				
		Time of Incubation			
	Pre-initial†	Initial	15 min.	30 min.	60 min.
0.01 M glusose	-	(1.8)	-	1.5	-
"	-	4.6	-	5.2	2.8
"	-	5.1	-	4.3	3.6
"	(1.2)	(1.5)	2.9	2.6	(1.5)
"	4.7	2.8	1.9	1.9	(1.4)
no addition	3.1	(1.2)	(1.4)	(1.6)	(1.4)
"	(0.6)	(0.6)	(0.4)	(0.3)	(0.2)
"	-	3.3	2.5	2.3	1.7
0.025 M pyruvate	-	(2.8)	(1.9)	(1.3)	(0.6)
"	-	-	2.7	2.3	2.3
"	-	2.6	-	-	1.7
"	-	2.7	-	-	1.7
"	(1.4)	(1.6)	(1.8)	(1.8)	(1.6)
0.025 M pyruvate in 0.11 M phosphate	(1.3)	(1.3)	(1.2)	(1.1)	(1.1)
"	-	(1.0)	-	-	(0.4)
"	-	(1.7)	-	-	(1.7)
0.04 M acetate	4.4	2.1	(1.3)	(1.5)	(1.2)

* Figures in parentheses refer to values below the accurate range of the method. This lower limit depends on the amount of the coloured compound in the volume of solution taken for reading, and may therefore vary widely when calculated as equivalent to one gram of tissue.

† Pre-initial values refer to determinations done on deproteinized solutions obtained from suspensions homogenized directly in a medium containing hydroxylamine, as described in the text.

FIGURE 4.

Hydrolysis of Acetyl Phosphate by Tissue Suspensions



One mg. of lithium acetyl phosphate in 0.5 ml. of either RP or 0.11 M phosphate, was added to 2 ml. of isotonic suspension containing 150 mg. of tissue per ml. The addition of hydroxylamine and the acyl phosphate determination were carried out on samples of the mixtures which had been incubated 0, 1, 2.5, 3.5 and 5 minutes at 38°.

After one hour's incubation, the amount of acyl phosphate in tissue suspensions in many cases fell below the lower limit of accuracy of the method, which is about 0.25 micromoles of acyl phosphate in the sample taken for determination. This corresponds to about 1.5 to 1.7 micromoles of acyl phosphate per gram of tissue, depending on the volume of solution taken for colorimetric reading. In several determinations the amount initially present per gram of tissue was less than this minimum amount.

From Table III it may be seen that all acyl phosphate values were very low and that most were near the lower limit of the method. There was no sign of an increase in acyl phosphate during incubation of the tissue suspensions with pyruvate, glucose or acetate, but usually a decrease in the amount present was observed, so the acyl phosphate found, even if it had been all acetyl phosphate, could not have accounted for all of the acetate which was found at the end of equivalent incubation times. The initial amounts of acyl phosphate found might account for the initial acetic acid values of 0.5-4.0 micromoles of acetic acid.

In the experiments in which glucose was added to the medium, the formation of phosphoglyceric acid must be taken into account. Phosphoglyceric acid is an intermediate in the glycolytic process of carbohydrate breakdown in tissues. It has the anhydride-with-phosphate type of structure which is capable of reacting with hydroxylamine to produce an hydroxamic acid, form a color complex with ferric chloride, and be read as acyl phosphate in the colorimetric method, Elliott et al. (20) showed that there was a rapid accumulation of lactate for a short period on the addition of glucose to brain tissue suspensions. In the course of this aerobic

glycolysis, probably appreciable amounts of phosphoglyceric acid would be produced, which, when converted to the hydroxamic acids by hydroxylamine, would be determined as a high initial acyl phosphate concentration.

It is clear from the foregoing that acetyl phosphate cannot account for any but a small portion of the acetate found when pyruvate or glucose are incubated with brain tissue suspensions. The source of this acetate formation must be found elsewhere.

In order to obtain other evidence concerning the possibility that acetyl phosphate is an intermediate in carbohydrate metabolism in brain, a single experiment was done to test the effect of added acetyl phosphate on oxygen uptake in brain suspensions homogenized in 0.11 M phosphate buffer. This high phosphate concentration should have inhibited the action of the acyl phosphatase present sufficiently to ensure the presence of an appreciable concentration of acetyl phosphate during the experiment. The addition of acetyl phosphate, however, had no effect on the oxygen uptake of the suspensions, (Table IV), whether it was added to a suspension which had been respiring in the presence of glucose, or to a suspension in which residual lactate had been reduced by a period of incubation without substrate.

Although brain suspensions in isotonic (0.11 M) phosphate with added pyruvate produced at least as much acetate (i. e. 11.5, 9.2, 8.9 µM per gram) as suspensions in Ringer-phosphate, no increase in acyl phosphate values was found in isotonic phosphate suspensions incubated with pyruvate (Table III). These observations support the conclusion, reached above, that it is unlikely that acetyl phosphate is an intermediate in the formation of acetate in these preparations.

TABLE IV

Oxygen Uptake of Brain Suspensions in the Presence of Acetyl Phosphate

Suspensions of whole rat brain homogenized in isotonic 0.11 M phosphate buffer with K and Mg. added were used. Three ml. of this suspension, 150 mg. of tissue per ml. were added to the vessels of Barcroft differential manometers containing alkali and filter paper rolls in the center well. 0.3 ml. of 0.11 M phosphate buffer solution containing 3 mg. of lithium acetyl phosphate were tipped into the vessels from side-bulbs at the times indicated. Gas phase air, temperature 38°.

Oxygen Uptake, cu. mm. O_2 per 450 mg. whole rat brain			
Additions	First 30 min.	Second 30 min.	Third 30 min.
0.01 M glucose	374	306	285
0.01 M glucose 3 mg. acetyl phosphate added at 30 minutes	380	315	300
No addition	262	203	135
No addition for first hour, 3 mg. acetyl phosphate added at 60 minutes.	274	188	143

Acetic Acid Formation and the Krebs Cycle

In their studies on the malaria parasite, Speck, Moulder and Evans (76) found that the Krebs cycle was concerned in the metabolism of acetate by their preparations. These authors found that while acetate production in the parasites was increased by high pyruvate

concentrations and in the presence of malonate, additions of catalytic amounts of the dicarboxylic acids decreased acetate formation.

It was decided to carry out similar experiments with isotonic brain tissue suspensions, to determine the role, if any, of the Krebs cycle in the acetate metabolism of brain tissue.

The results of a series of experiments on the formation of acetate in the presence of malonate are given in Table V.

TABLE V

Acetic Acid Formation From Pyruvate and Glucose by Isotonic Brain Suspensions in the Presence of Malonate

Results are expressed as micromoles of acetic acid formed per gram of whole rat brain in one hour's incubation at 38°. All figures are corrected for the average amount of acetate found initially. (see Table II).

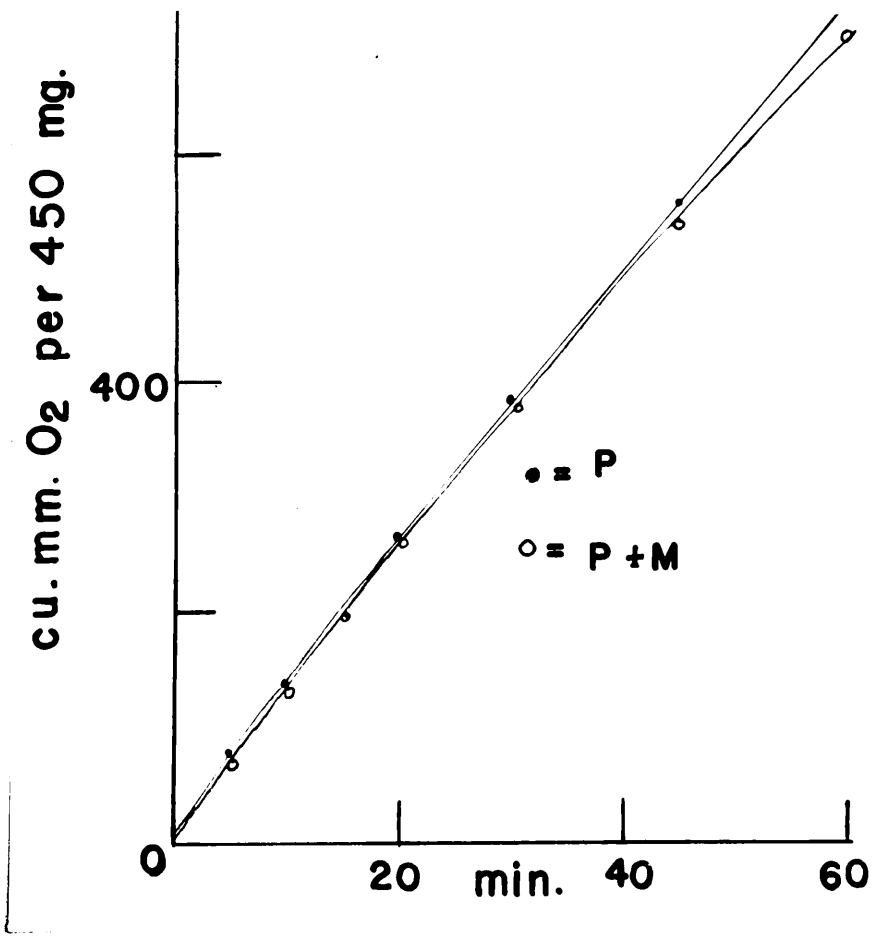
Micromoles of Acetic Acid Formed in 1 Hour		
Additions		Micromoles
0.025 M pyruvate	0.02 M malonate	12.1
" "	" "	11.0
" "	" "	16.7
" "	" "	14.6
" "	" "	13.4
" "	" "	
	Average	13.5
0.01 M glucose	0.02 M malonate	3.9
" "	" "	5.2
" "	" "	1.1
	Average	3.4

Although malonate in the 0.02 M concentration used, only inhibited oxygen uptake in the presence of pyruvate by

about 5 % (see Fig. 5), it increased the formation of acetate from pyruvate by about 100%. It did not increase acetate formation from glucose. However, alpha-ketoglutarate when added with pyruvate in experiments done in isotonic and hypotonic suspensions increased acetate formation in both cases, (Table VI). This finding is different from that of Speck et al. , who found that the addition of dicarboxylic acids to their malaria prasite preparations decreased acetate formation. Citrate and malate, when added to brain suspensions with pyruvate, did not appear to markedly effect the formation of acetate, although when added to brain suspensions in the absence of pyruvate they did decrease acetate production somewhat. (Table VI)

FIGURE 5

Effect of Malonate on the Respiration of Isotonic Brain Suspensions in the Presence of Pyruvate.



Suspensions of whole rat brain homogenized in isotonic Ca-free Ringer-phosphate were used. Pyruvate and malonate solutions were added to the vessels of Barcroft manometers before the introduction of 3 ml. of the suspension (150 mg. tissue/ml.). Final concentrations of substances; pyruvate 0.025 M, malonate 0.02 M. Gas phase air, temperature 38°.

Since malonate is an inhibitor of succinic dehydrogenase and would block the function of the Krebs cycle by inhibition of this key enzyme, the increase in acetate formation from pyruvate observed in these suspensions, on incubation of tissue suspensions in the presence of malonate and pyruvate, might indicate that acetate metabolism in brain is at least partially concerned with this cycle.

TABLE VI

Formation of Acetate by Rat Brain Suspensions in the Presence of Krebs Cycle Intermediates, in the Presence and Absence of Pyruvate.

The results are expressed as micromoles of acetic acid formed per gram of whole rat brain in one hours' incubation at 38°. The figures are corrected for initial values as usual.

Additions	Micromoles of Acetic Acid Formed in 1 Hour	Average
0.025 M pyruvate		6.2 *
0.025 M pyruvate + 0.005 M alpha-ketoglutarate	9.1,10.6,12.3	11.5
0.025 M pyruvate + 0.005 M malate	9.1,11.8,5.5,10.7	9.3
" " + 0.005 M citrate	4.3	
" " + 0.005 M glutamate†	5.0	
No addition		3.2 *
0.01 M citrate	0,0	
0.02 M malate	1.3,1.7	
0.02 M glutamate	1.0,2.7	

† Glutamic acid is included as a Krebs' cycle intermediate since it is in equilibrium with alpha-ketoglutaric acid in the mammalian body.

* from table II.

Formation of Acetate By Hypotonic Suspensions

Speck et al. (76) in the malaria parasite studies referred to previously, showed that free parasites produced much more acetic than parasitized erythrocytes. They considered that the free parasite preparation constituted an abnormal condition for the parasite. It was felt that cytolysed tissue might represent an analogous abnormal condition for brain tissue and an increased acetate formation might be exhibited. By preparation of the suspension in hypotonic medium, cytolysis, or at least, disruption of respiratory mechanisms is more marked than in isotonic suspensions.

The medium for the preparation of hypotonic suspensions was made by substituting distilled water for the 0.9% NaCl solution used as the basis of Ca-free Ringer-phosphate. All other constituents of the medium (i. e. $MgSO_4$, KCl etc.) were added in the same amounts as in RP.

Hypotonicity of suspensions resulted in a small increase in acetate production, presumably caused by the disruption of the enzymic mechanisms concerned in the efficient utilization of the added substrates. Table VII compares and summarizes acetate values obtained with isotonic and hypotonic suspensions in the presence of the same added substrates.

Other Factors: Effect of Magnesium Ions, Thiamine, and Fluoro-acetate on Acetate Formation

From investigations carried out with enzymes from various sources, it is known that diphosphothiamine acts as a coenzyme for the oxidation, decarboxylation, dismutation and other reactions of pyruvate in biological systems (67).

Magnesium ions are known to be necessary for the activity

TABLE VII

Effect of Various Factors on Acetate Production

Micromoles of Acetic Acid Per Gram

Incubation 1 Hour at 38°

Isotonic Suspensions		Hypotonic Suspensions	
Additions	Acetate Formed*	Additions	Acetate Formed*
Pyruvate 0.025 M	6.2 (Average)	Pyruvate 0.025 M	9.0,6.5,7.5, 14.2
Pyruvate & Malonate 0.02 M	12.1,11.0,16.7, 14.6,13.4	Pyruvate & Malonate 0.02 M	13.2,13.9
Glucose 0.01 M	3.2 (Average)	Glucose 0.01 M	3.1,9.6,5.0
Pyruvate & Malate 0.005 M	5.5,10.7,9.1 11.8	Pyruvate & Malate 0.005 M	7.5,8.1
Pyruvate & α-Keto-glutarate 0.005 M	10.6,12.3	Pyruvate & Malate & Malonate	9.5,10.6

*After deducting average initial figure.

of the purified enzyme preparation of Green et al.(27), isolated from heart muscle, which attacks pyruvic and other keto-acids.

The results of experiments on the production of acetate from pyruvate in the absence of added magnesium or with five times normal concentration of magnesium, and on the addition of thiamine in the presence of pyruvate, are presented in Table VIII.

TABLE VIII

Acetic Acid Formation From Pyruvate in Isotonic Rat Brain Suspensions with Varying Amounts of Magnesium Ions Present, and In the Presence of Added Thiamine.

Results are expressed as micromoles of acetic acid formed per gram of whole rat brain, in one hour's incubation at 38°. The figures are corrected for initial values, as usual.

Micromoles of Acetic Acid Formed in 1 Hour	
Additions	Micromoles
0.025 M pyruvate - Mg omitted from suspension	4.5
" " " " " "	5.5
" " " " " "	
" " + 0.005 M Mg in suspension†	4.7
" " + 0.003 M thiamine	11.9,6.5
	9.4,10.1

†The normal concentration of Mg. in RP is 0.001 M.

The omission of Mg from the medium in which the brain suspension was made, had no effect on acetate production. Presumably either Mg. is not necessary for the formation of acetate from pyruvate in these preparations, or the amount of Mg. contained in the tissue itself is sufficient for this aspect of pyruvate catabolism.

Thiamine when added to the suspensions produced an increase in acetate formation. The mechanism of this effect is not understood although it probably involves formation of cocarboxylase which subsequently enters into the metabolic reactions of pyruvate.

Fluoroacetate. Sodium monofluoroacetate has been mentioned earlier, variously as a specific inhibitor of pyruvate and of acetate

oxidation. The findings of experiments on acetate production from pyruvate and from glucose, in the presence of fluoroacetate, are indicated in Table IX.

TABLE IX

Acetic Acid Formation From Pyruvate and Glucose in the Presence of Fluoroacetate

Additions	Acetic Acid Formed in 1 Hour per Gram of Tissue micromoles formed*		
	With Tissue	Blanks	Average Difference
0.025 M pyruvate + 0.02 M fluoroacetate	29.5,15.6,12.5	11.1,13.3,8.4	8.3
0.01 M glucose + 0.02 M fluoroacetate	11.7,11.4	-	0.7**
0.025 M pyruvate + 0.10 M fluoroacetate	27.5	18.6,28.2	4.1
0.01 M glucose + 0.10 M fluoroacetate	22.2	-	0 **

*The average initial acetic value (1.6 µM/gm.) has been subtracted.

**The pyruvate-fluoroacetate blank of corresponding concentration has been subtracted.here.

Fluoroacetate[1] had little effect on the rate of respiration of suspensions in the presence of pyruvate or glucose (Fig. 6) and it seems as if it does not interfere with the metabolism of these rat brain suspensions, since no appreciable increase in acetic acid formation seemed to occur. Fluoroacetate, when present in solutions analysed for acetate by the steam distillation method used in this study, gives rise to a considerable amount of titratable substance which is estimated as acetic acid.

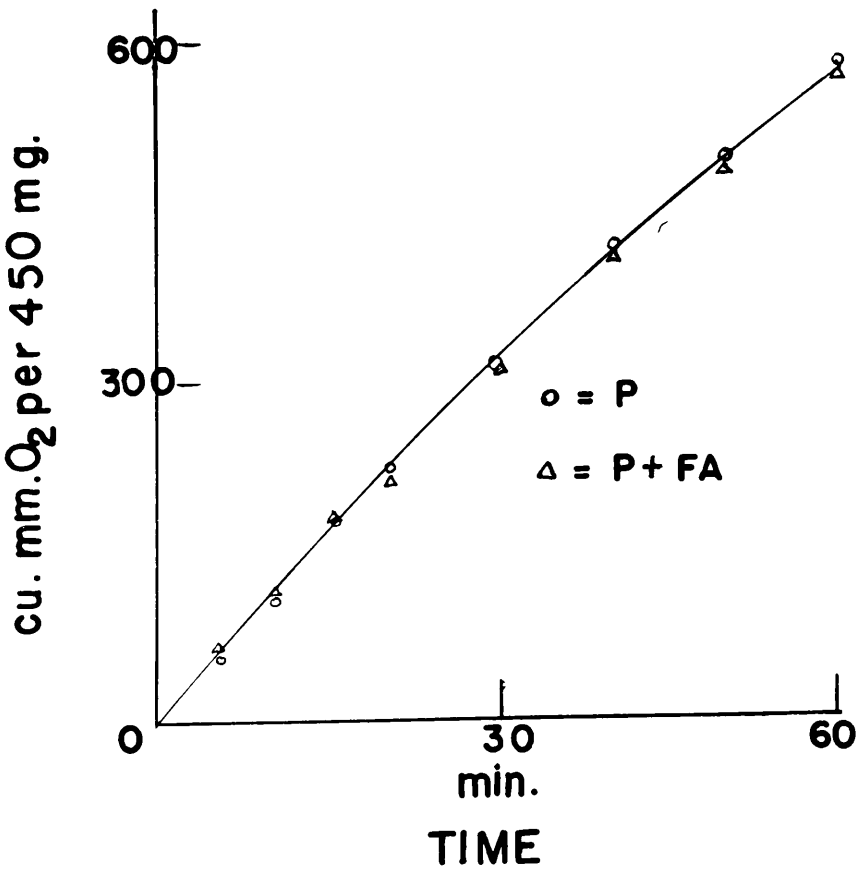
.....

[1] The sodium monofluoroacetate used in these studies was a sample kindly donated by Dr. E. S. G. Barron.

These large and varying blank values make it difficult to state definitely that the addition of fluoroacetic acid has any real effect on acetate formation in rat brain tissue suspensions.

FIGURE 6

Respiration of Isotonic Brain Suspensions in the Presence Pyruvate and Fluoroacetate



Final concentrations of added substances were: 0.025 M pyruvate, 0.02 M fluoroacetate. Respiration was followed manometrically as described in Fig. 5.

Presumably the substance interfering in the acetic acid determination is fluoroacetate itself, despite its high boiling point (169°), and not acetate formed by an enzymic spliting off of the fluorine atom in the presence of tissue. Fluoroacetate gives a high titration value whether it is added directly to the distillation flasks and distilled, or is shaken for one hour at 38° in Ringer-phosphate (RP), in the absence of tissue, and then distilled.

Source of the Acetate Formed by Brain Suspensions in the Presence of Pyruvate

The acyl phosphate found in brain tissue was not present in large enough quantities to account for the amounts of acetate formed by brain suspensions in the presence of pyruvate, and the addition of the dicarboxylic acids of the Krebs cycle seemed to affect acetate formation but little when added to suspensions under these conditions. It was decided to investigate the source of the acetate formed more closely.

It is known that on warming a solution of pyruvic acid to 50° in the presence of Na_2CO_3, a considerable amount of acetic acid is produced. It seemed possible that some acetate might be formed at 38° in the faintly alkaline suspension medium. Therefore pyruvate was incubated in Ringer-phosphate solution, in the absence of tissue, to determine the amount of acetate formed spontaneously from pyruvate under the same experimental conditions used in tissue incubation. In Table X the results of such experiments are reported.

Appreciable amounts of acetic acid were formed from 0.025 M pyruvate when incubated in Ringer-phosphate. Deduction of the 2.2 micromoles of acetate which was found to be formed

spontaneously from pyruvate, from the amount of acetate found after incubation of tissue in the presence of pyruvate, would considerably reduce the amounts formed by factors in the tissue.

Possible role of Ascorbic Acid in Acetate Formation by Brain Tissue

It was reported by Cavallini (11) that ascorbic acid was capable of bringing about oxidation of pyruvate to acetic acid and carbon dioxide in the acid to neutral pH range. Ascorbic acid oxidized pyruvate at all pH's studied, acetate being formed only in the acid to neutral pH range. No mechanism for this reaction was suggested in the abstract available to the writer.

Ascorbic acid, in solution, is readily oxidized to dehydroascorbic acid in the presence of traces of copper. Possibly dehydroascorbic acid oxidizes the pyruvate and becomes reduced to ascorbic acid, which may then be reoxidized by oxygen. Alternatively, hydrogen peroxide, produced in the oxidation of ascorbic acid, reacts with the pyruvate. Pyruvate is known to be oxidized by hydrogen peroxide, without catalysts, to produce acetic acid and carbon dioxide (65).

It was thought that it would be of interest to determine the order of magnitude of the amount of acetic acid, or substance estimated as acetic acid, which could be produced from pyruvate by the amount of ascorbic acid present in brain tissue.

Varying concentrations of neutralized ascorbic acid and sodium pyruvate were added to Ringer-phosphate, shaken at 38° for the usual one hour period, and acetic acid determinations carried out at the end of that time.

In order to see if the traces of copper present in the distilled water used in making up the reagent solutions had

any function in the reaction of ascorbic acid and pyruvate in the absence of tissue, several experiments were done with twice glass-distilled water instead of the usual Ringer-phosphate medium. This procedure had no effect on the reaction.

In Table X the results of a number of experiments done with varying concentrations of ascorbic and pyruvic acids, with and without tissue present, are reported.

TABLE X

Non-Enzymic Formation of Acetate From Pyruvate

Incubated for 1 hour at 38°, pH 7.2-7.4.

Medium	Pyruvate added µM/ml.	Ascorbate added µM/ml.	Acetate Formed µM/ml.	Equivalent µ per G. of ti Average
Ringer-phosphate	25	-	0.70,0.36,0.23,0.05	2.2
"	25	15	3.4,4.1	25.1
Tissue Suspension	25	15	3.6,2.7	20.7
Ringer-phosphate & 0.02 M Malonate	25	15	4.9	29.8
Ringer-phosphate	12	30	7.9	47.6
"	25	0.3	0.2,0.4,0.6	2.7
"	1.4	-	0.3,0.18,0.12	1.0
"	1.4	0.3	0.11,0.12,0.25,0.63	1.9

From Table X it will be seen that appreciable amounts of a substance estimated as acetic acid are produced from ascorbic acid and pyruvate when they are incubated together, at all concentrations.

There was no more "acetate" formed from 0.025 M pyruvate and 0.015 M ascorbate in the presence of tissue, than was formed when these substances were incubated in Ringer-phosphate alone. It is therefore unlikely that enzymic mechanisms affect the reaction. Ascorbic acid itself, when incubated with or without tissue

suspension, gives rise to a slight amount of some substance estimated as acetic acid. However the sum of the blanks found on the incubation of pyruvic and ascorbic acids separately, does not account for the large amount of acid found when both of these substances are incubated together.

The ascorbic acid content of normal rat brain was found by Bessey and King (6) to be 0.36 mg. of ascorbic acid per gram of whole brain. A brain suspension made up to a concentration of 150 mg. of tissue per ml. would thus contain 0.0003 M ascorbic acid, (0.5 μM/ml.)

The sum of pyruvic and lactic acids, found initially in homogenates of rat whole brains with no added glucose, was found by Elliott et al. (20) to be 0.82 mg. per gram of brain tissue. If a brain suspension was made up to 150 mg. per ml. concentration, the initial concentration of pyruvic acid (counting lactic acid also as pyruvic for this purpose) would be 0.0014 M. (1.4 μM/ml.) These concentrations were used in some of the experiments reported in Table X. They gave rise to a small amount of acetic acid when incubated in RP in the absence of tissue.

From these blank values obtained with these "tissue concentrations" of ascorbate and pyruvate, (Table X) one can see that a large fraction of the acetic acid produced on incubation of tissue with glucose, or with no added substrate, could come from this reaction of ascorbate with the pyruvate already present in the tissues. The blank values found with the usual (0.025 M) concentration of added pyruvate and tissue concentration of ascorbate, also indicate that when tissue is incubated with 0.025 M pyruvate, some of the acetate formed could have been formed by reaction of tissue ascorbate with some

of the added pyruvate.

In Table XI the average values for acetate found when tissue is incubated with pyruvate, glucose and with no added substrate, minus the blanks found on incubation of the relevant high and low concentrations of pyruvate, and the tissue concentrations of ascorbate are presented.

TABLE XI

Values For Acetate Formation After Incubation Blanks of Pyruvate, and Ascorbate Plus Pyruvate, Are Substracted.

All results are expressed as micromoles of acetate per gram of brain tissue, or an equivalent amount of RP in the case of the blanks. The pyruvate and "tissue concentration" ascorbate incubation blank has only been subtracted from the average value for acetate formed in the presence of pyruvate. The average initial acetate value has been subtracted from all values for tissue.

Micromoles of Acetic Acid Formed in 1 Hour/gram of Tissue			
Blanks to be subtracted	0.025 M pyruvate	0.01 M glucose	no added substrate
Formed in 1 hour (averages)	6.2	3.2	3.2
Pyruvate and "tissue ascorbate incubation blank	2.7		
Tissue concentration pyruvate and ascorbate		1.9	1.9
Balance, acetic acid formed in 1 hour	3.5	1.3	1.3

From Table XI it will be seen that the amounts of acetate left after the relevant blanks have been subracted are still appreciable, and in view of the effects of malonate and thiamine in increasing the amounts of acetate formed from pyruvate and glucose by brain tissue suspensions, it would be difficult to ascribe acetate formation in these preparations wholly to non-

enzymic mechanisms. Certainly a portion of the acetic acid formed may be the result of the non-enzymic mechanisms described in this last section, but since malonate is known to interfere with the functioning of the Krebs cycle, and thiamine to be an essential component of the coenzyme cocarboxylase, it would appear from the effect of these substances, that enzymic tissue mechanisms have some role in acetate metabolism in brain, even though the accumulation of acetate in these preparations may be an artefact due to <u>in vitro</u> conditions.

Summary

1. Observations have been made on methods for the determination of acetate and acyl phosphate in tissue suspensions.
2. Previous work on the formation of acetate by respiring brain suspensions, especially in the presence of added pyruvate, has been confirmed.
3. No evidence could be obtained that the substance formed is actually acetyl phosphate, which breaks down to yield acetic acid in the course of the estimation, or enzymatically during the incubation of the tissue.
4. The amount of acetate formed in brain suspensions is increased by the presence of malonate and by homogenization in hypotonic medium. It seems also to be increased by the addition of malate or α-ketoglutarate and by thiamine.
5. Spontaneous breakdown of pyruvate could account for part of the acetate formed from pyruvate added to tissue.
6. Fluoroacetic acid interferes with the determination of acetic acid. There was no evidence that it increases the formation of acetate by brain tissue suspensions. It does not affect the oxygen uptake of these suspensions with glucose or pyruvate as substrate.

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