THE EFFECT OF NITROUS OXIDE ON ENZYME SYSTEMS

by

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Thesis

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
ADP	Adenosinediphosphate
AMP	Adenosinemonophosphate (Adenosine-5-phosphate)
ATP	Adenosinetriphosphate
ATP-ase	Adenosinetriphosphatase
CoA	Coenzyme A
DPN	Diphosphopyridine nucleotide
DPN-ase	Diphosphopyridine nucleotidase
HDP	Hexose diphosphate
PL	Pigeon Liver Extract
RBH	Rat Brain Homogenate
TPN	Triphosphopyridine nucleotide

INTRODUCTION

Ever since the anesthetic and analgesic properties of nitrous oxide were discovered, the question of how nitrous oxide produces these effects has constantly arisen. Some workers claim that, since nitrous oxide is an inert and nontoxic gas, it does not influence the metabolic processes of body tissues. Others, however, feel that the anesthetic powers of nitrous oxide are either attributable to or accompanied by, inhibitory effects on the oxidative processes of the nervous system.

This study was undertaken with the view to finding out if nitrous oxide directly affects the metabolic mechanism of the tissue cells, and if so which enzyme systems are most sensitive to its action.

HISTORY OF ANESTHESIA

As far back as history records, man has been searching for methods to relieve pain. Primitive man depended for relief upon priestesses, sorcerers, conjurers, or medicine men, who cast spells, and uttered incantations, to drive out the demons who were believed to be responsible for man's suffering. Later these practitioners made use of concoctions made from roots, barks, herbs and various plants to produce unconsciousness or artificial sleep. The ancient Chinese and the Greeks used opium and mandragora. The early Egyptians and Arabians administered the fumes of smoking hemp or hashish, which produced mental stimulation followed by sleep. They also employed compression of the carotid arteries to produce temporary unconsciousness. Compression of both nerves and blood vessels in the area for operation, a practice introduced in the 17th Century, was the first attempt at producing regional anesthesia. The actual beginning of inhalation anesthesia was marked by the practice of soaking a sponge in a concoction of various volatile drugs and holding it to the patient's face. The era of modern anesthesia, however, really began with the discovery of nitrous oxide by Priestley, in 1772.

THE HISTORY OF THE USE OF NITROUS OXIDE IN ANESTHESIA

Soon after Priestley's discovery it was found that

the inhalation of nitrous oxide produced pleasurable sensations and strange mental behaviour. The sensations experienced and the amusing behavioural effects manifested after the inhalation of the gas afforded a popular means of entertainment. The tendency of the subject to pass through a stage of laughter before losing consciousness, led to the use of the name "laughing gas", by which it is still commonly known. During these exhibitions people had fallen and sustained painless injuries. It was this fact that led Sir Humphrey Davy to carry out experiments with nitrous oxide.

In 1799 he obtained pure nitrous oxide and proceeded to inhale it for three to four minutes. Always when breathing nitrous oxide he experienced the intensification of visual and auditory perception and a

> "sensation analogous to gentle pressure on all muscles, attended by a highly pleasurable thrilling, particularly in the chest and the extremities... The senses of muscular power became greater, and at last an irresistible propensity to action was indulged in.' But 'whenever its operation was carried to its highest extent, the pleasurable thrilling... gradually diminished, the sense of pressure on the muscles was lost; impressions ceased to be perceived, vivid impressions passed rapidly through the mind and voluntary power was altogether destroyed, so that the mouthpiece generally fell from my unclosed lips." (1).

On several occasions of breathing nitrous oxide, Davy experienced an analgesic state which led him to try breathing the gas when a headache or toothache was bother-

ing him (1). All these observations made Davy suggest the use of nitrous oxide to relieve pain during surgery. However his suggestions received little attention.

Henry Hill Hickman, in 1820, was the first to use nitrous oxide successfully to relieve pain during operations on animals. But he was not able to impress the medical profession, and even to the time of his death in 1829 was unable to persuade surgeons to use the anesthetic on human subjects.

Horace Wells in 1884, a dentist in Hartford, Conn. was the first to use nitrous oxide in dentistry. Referring to nitrous oxide he remarked, "I believe that a man may be so drunk with this gas or some similar agent, that dental or other operations may be performed on him without any sensation of pain." (2).

Wells tried it on himself first when he was to have a tooth extracted and discovered that he felt no pain. He was so certain of the promise of his discovery and was so confident of the future success of its use, that he and other dentists began using nitrous oxide in their practice. This was really the first practical demonstration of anesthesia.

Wells selected nitrous oxide rather than ether, a drug more easily given for surgical operations, because it was safer, more respirable and less liable to cause illness to the patient. Time has since vindicated his ideas but

nitrous oxide proved to be a difficult anesthetic to administer. His failure to convince the authorities at the Harvard Medical School led to the abandonment of the gas as an anesthetic for surgical operations, although it retained some of its popularity among the dentists.

Nitrous oxide anesthesia advanced only when improvements were made in the construction of a suitable apparatus for its administration. Wells used a rubber bag, and made his patients breathe into and from it.

G.Q. Colton, in 1862, reintroduced nitrous oxide into dental practice in America. He designed an apparatus for the administration of the gas, and demonstrated its use in Paris in 1867. He succeeded in interesting leading Parisian dentists in its use. Among them was T.W. Evans who later introduced the practice of nitrous oxide anesthesia into England.

In 1868, E.A. Andrews reported the use of oxygen with nitrous oxide. The use of oxygen as an adjunct was a very important finding, because it made possible the maintenance of anesthesia for an extended period of time (3). He recommended the use of oxygen and nitrous oxide in the volume ratio of 1 : 5 which is the ratio of oxygen to nitrogen in air.

At the same time Paul Bert in Paris, was trying to interest French doctors in the use of nitrous oxide and

oxygen mixtures under pressure. The method of administering such a mixture was not easy, and only a few surgeons actually tried it. Bert tried to solve this problem and after carrying out numerous experiments on animals, he came to the conclusion that mixtures of oxygen and nitrous oxide in proportions similar to those in which nitrogen and oxygen are found naturally together in air could be used to produce anesthesia and maintain it for longer periods. This proved successful and his idea was gradually accepted.

After the introduction of the practice into Great Britain in 1868, nitrous oxide remained the anesthetic of choice for short dental operations. However, anesthetists continued to administer it free of any admixture of air. Between 1885 and 1897, F.W. Hewitt devised various improvements in the apparatus used for the administration of nitrous oxide and oxygen and experimented on the merits of various mixtures of these gases. With continuous improvements in the apparatus the administration of nitrous oxide has been made easier and its use extended.

E.I. McKesson believed in the scope and utility of nitrous oxide-oxygen anesthesia and analgesia and he devoted his life to an intensive concentration on that subject, which enabled him eventually to bring his method of pain relief to practically ultimate perfection. He perfected the method and the apparatus for administering nitrous oxide-

oxygen gas mixtures, basing the improvements on requirements of technical experience. He used nitrous oxide-oxygen for almost every kind of operation. Since his death in 1935, his techniques and apparatus have been widely used in the hospital in Toledo where he worked. The practice has also been adopted by many of the leading anesthetists.

PROPERTIES OF NITROUS OXIDE

Nitrous oxide is a colourless, tasteless, non-irritant gas with a faint, sweetish odour. It boils at -88.7°C. It is soluble in alcohol and water.

The great advantage of nitrous oxide in anesthesia is its high solubility in body fluids, and stability in living tissue (4). According to Kety *et al.* (5) the solubility of nitrous oxide in whole blood is directly proportional to the red blood cell content. Nitrous oxide dissolves in blood to an extent of 45% by volume as compared to oxygen with a solubility of only 18.5%. - only 0.24% of the 18.5% is contained in the serum, the rest being held in the red blood cell in the form of oxyhaemoglobin (4). In the case of nitrous oxide however, 29% is contained in the serum. Thus when blood is saturated with nitrous oxide, the serum contains more than 100 times as much nitrous oxide as oxygen. (4). It is about 15 times as soluble as nitrogen in blood. The solution of nitrous oxide in the human or dog brain is 0.437 cc. per gram of brain tissue (5).

Nitrous oxide is prepared by heating salt ammonium nitrate in a closed retort.

$$NH_4NO_3 \rightarrow N_2O\uparrow + 2H_2O$$

Nitrous oxide thus generated contains impurities such as ammonia, oxides of nitrogen, oxygen and carbon dioxide. It is purified and stored in cast iron cylinders in the liquid state.

ANESTHESIA

The word "anesthesia" comes from the Greek meaning insensibility. H.K. Beecher calls anesthesia and narcosis "conditions in which the normal response to stimuli of the whole or part of the body are temporarily depressed." He says that the two conditions are probably manifestations of the same general process, but we cannot assume that they are identical. The term narcosis should be reserved for reversible reactions in single cell organisms or groups of cells from higher organisms. "Anesthesia may be responsible for the complicated reversible depression of the senses or automatic activity of higher forms of life. In man the primary characteristic of general anesthesia is loss of consciousness. The reversible nature of the process is one of its outstanding characteristics." (5a).

E.I. McKesson, on the other hand, defines true anesthesia as a state due to anoxemia or a depression of the

metabolism of certain brain and nervous tissues. He believes that "anesthesia approaches the control of life processes so intimately that it may be observed without destroying delicate balances." (4).

THEORIES OF NARCOSIS

Many theories have been propounded to explain the mechanism of narcosis. The following are among the ones more commonly held.

1. Lipoid Theory (6)

This theory was put forward by Meyer and Overton. They found that anesthetic drugs have a high solubility in and a special affinity for lipoid tissue, which is found in abundance in the nervous system. They, therefore, postulated that narcosis was caused by the interference of the drug with cell metabolism. This is probably the most generally accepted theory at the present time (for lipoid soluble agents).

2. Surface Tension Theory (7)

Traube attributed the narcotic action of certain drugs to their effects on the surface tension of the cell. These drugs lower the surface tension of the cell which results in a change in cell permeability and subsequently interferes with the respiration of the cell.

3. Adsorption Theory (8)

Warburg explains the action of narcotics by their adsorption on cell surfaces, thus causing a change in the permeability of the cell. He bases this explanation on the fact that narcotics are readily adsorbed on surfaces *in vitro*.

4. Chemical Combination Theory (9)

This theory was put forward by Moore and Roaf who stated that the drug combines with cell proteins to form unstable compounds with an alteration in the cell metabolism.

5. Protein Coagulation Theory (10)

Bernard claims that protein coagulation results from the action of the narcotic drug - this action is, however, reversible.

6. Tissue Oxidation Theory (11)

This hypothesis states that the oxidative activity of nerve tissue is inhibited by the action of the anesthetic drug resulting in a depression of the metabolism and consequent narcosis.

THEORIES OF THE ANESTHETIC ACTION OF NITROUS OXIDE

To explain the specific anesthetic effect of nitrous oxide, three main theories have been advanced.

1. Theory of Hyperoxygenation (12)

This theory postulated by Sir Humphrey Davy in 1799 is merely of historical interest as it was discarded quite soon after nitrous oxide anesthesia came into use.

Davy believed that nitrous oxide was broken down in the blood to nitrogen and oxygen. The excess of oxygen thus produced gave rise to an overproduction of carbon dioxide with a resultant "interval asphyxia". This theory was rejected when it was discovered that nitrous oxide is very stable.

2. Oxygen Exclusion Theory (13)

The second theory explaining the action of nitrous oxide centered about the fact that nitrous oxide is very highly soluble in the blood. Some anesthetists erroneously believed that so many molecules of nitrous oxide were present in the blood that less oxygen could dissolve and that anesthesia was a consequence of this dilution of oxygen.

The most susceptible or highly specialised cells, those of brain tissue, are the first to show the effect of oxygen want. This is evidenced by the impairment of the most highly specialised functions such as memory, reasoning, association, and consciousness, followed by drowsiness, loss of coordination and the production of anesthesia. At the same time, however, the supply of oxygen may be quite adequate to maintain the more stable autonomic functions of the other

organs of the body.

E.I. McKesson believed that anesthesia was a result of the decreased oxygenation of the nervous system and, therefore, to a certain extent was a consequence of anoxemia (4).

This theory is no longer accepted as it has been shown by various workers that anesthesia with nitrous oxide can be obtained without anoxemia and that oxygen lack may interfere with the production of anesthesia (14).

3. Specific Action Theory (13)

This is the theory presently held by most anesthetists. Nitrous oxide of itself does exert a weak anesthetic action, and when administered with less than atmospheric oxygen, the mild anesthetic action of the gas may be enhanced to produce true or surgical narcosis. "The action of nitrous oxide on the brain consists of primary stimulation of psychic areas, which are later depressed in insensibility." (15).

Many factors favour this theory. A mixture of 90% oxygen and 10% nitrous oxide will produce an analgesic state (13). This means that the analgesia must be caused by the nitrous oxide even when present in such a small quantity.

If the anesthesia is a result of oxygen exclusion, then a mixture of the two gases that contains more than 20.9% oxygen should not produce narcosis. However, in some cases, the percentage of oxygen can be raised considerably above that

while still maintaining anesthesia. During prolonged nitrous oxide - oxygen narcosis, it is necessary to gradually increase the percentage of oxygen in the mixture to obtain proper anesthesia (13).

Zelnigher (16) states that according to the gas laws the rates of diffusion, solubilities in the blood, diffusion through the permeable alveolar membrane, diffusion in the lung, and the absorption coefficients in the blood of oxygen and nitrous oxide are independent of one another. He summarises his views thus:

> "Therefore the two systems (oxygen and nitrous oxide) are distinct entities, nitrous oxide exerting its effect only as an anesthetic agent totally unrelated to oxygen behaviour. The concept that oxygen is crowded out by nitrous oxide is fallacious."

Goodman and Gilman (17) state that an 80/20 $N_2O:O_2$ mixture may produce anesthesia in some patients. If nitrogen is substituted for nitrous oxide in such a mixture, anesthesia ceases rapidly. Even if oxygen is reduced to 10%, no anesthesia occurs with the nitrogen, but it is produced with a 90/10 mixture of N_2O - O_2.

STAGES OF ANESTHESIA

The course of nitrous oxide anesthesia may be divided into the following four stages depending on the concentration of the anesthetic in the blood (4). These stages merge into each other and it is not always possible to determine precisely where one ends and another begins.

1. Analgesia

This stage comprises various degrees of insensibility to pain whilst the patient is still conscious. If desired this stage may be maintained for some time. If the induction for anesthesia is properly conducted this stage is very brief.

2. Unconsciousness with Exaggerated Reflexes

In this stage the patient is completely unconscious. The exaggeration of the reflexes is probably attributable to a paralysis of the inhibitory centres and not to direct stimulation by the anesthetic itself. These reflexes can be avoided or controlled by the proper induction of anesthesia.

3. Surgical Anesthesia

(a) Light Plane

The signs of light anesthesia are those associated with more or less active reflexes. The respiration, which is the most important and dependable sign in N_2O - O_2 anesthesia is quiet, shallow, slow and regular. The pulse rate and the cardiac output tend to increase slowly, particularly if the patient did not receive premedication. The blood pressure remains normal.

(b) Normal or Surgical Plane

This plane of anesthesia is characterised by the absence of active reflexes or convulsive movements, and by the production of generalised relaxation. The breathing tends to be regular, being full or adequate in volume, and uniform in rate though somewhat faster than normal. The pulse rate is slower than in the light plane, but it is still somewhat faster than normal. The blood pressure remains normal.

(c) Profound Plane

This plane is marked by certain muscular movements which are spastic, clonic or tetanic in character. The respiration gradually becomes quiet, shallow, slow and irregular. Consequently it is insufficient and constitutes a distinct danger signal. The pulse rate is at first accelerated well above normal and the cardiac output increases. Then as the fourth stage approaches the pulse rate becomes slower and irregular, often being scarcely perceptible, indicating a failing circulation. Accordingly the blood pressure rises slightly at first and then falls abnormally.

4. Bulbar Paralysis with Cessation of Respiration

This stage is a continuation of the profound plane. The acute or prolonged oxygen shortage will finally result in complete cessation of respiration. The heart continues

to function for one or two minutes, during which interval oxygen must be forced into the lungs to resuscitate the patient. The blood pressure in this stage becomes imperceptible because of the failing circulation.

CYANOSIS AND NITROUS OXIDE ANESTHESIA

The depth of the anesthesia is controlled by the proportion of N_2O - O_2 administered (13).

Moderate cyanosis is not regarded as a serious omen in nitrous oxide anesthesia since signs indicating good anesthesia and safety for the patient may accompany it (4). The colour of the blood is not a reliable guide because it bears no relation to the depth of narcosis. Since oxygen in blood is replaced to a great extent by nitrous oxide it is impossible to produce anesthesia in most patients and maintain a pink skin and oxygenated blood (13). The colour of the blood is determined by the relation between the oxygen absorbed and the total hemoglobin in the body. The degree of cyanosis tends to be pronounced in the plethoric type of patient and is associated chiefly with induction and the earlier stages of anesthesia. Anemic patients will not exhibit symptoms of cyanosis even in the profound plane. Cyanosis bears no relation to the depth of anesthesia (13).

A patient may become cyanotic during N_2O - O_2 anesthesia without any depression of respiration or circulation which normally occurs in hypoxia. However, anemic patients may

become hypoxic without becoming cyanotic. The presence of cyanosis in N_2O - O_2 anesthesia is not necessarily a sign of danger, nor is its absence a sign of safety (13).

HYPOXIA

Hypoxia is that stage in which there is an oxygen deficiency in the tissues (13). The body has a remarkable tolerance and capacity for adaptation to hypoxia. There is an "essential minimum" requirement of oxygen, and only if the oxygen supply is reduced below this critical level does the system suffer from hypoxia. This applies also in anesthesia. The symptoms exhibited during acute oxygen deprivation, are comparable to those occurring in the profound plane of N_2O - O_2 anesthesia. It is possible to save a patient in this condition, whose heart is still beating, by the administration of 100% oxygen (13).

Short periods of oxygen deprivation do not produce any after-effects. More profound exposures to oxygen want cause headache, nausea and mental depression. Prolonged deprivation may cause severe and even fatal results. Similar results may be caused by breathing pure nitrous oxide (13).

During N_2O - O_2 anesthesia there may be danger of hypoxia in the stage of induction and of profound narcosis (13).

During the induction of anesthesia the oxygen content

of the gas mixture is usually adjusted to a minimum, or may be reduced to zero. During this stage the patient must be watched carefully, and upon the first sign of oxygen lack, which is usually indicated by an increase in the respiratory rate, the oxygen must be increased until breathing becomes normal. If oxygen is not administered the patient will rapidly pass into the profound plane of narcosis.

It is much more difficult to detect hypoxia during prolonged narcosis and this stage is therefore more dangerous. The development of hypoxia, accompanied by falling blood pressure and an increased pulse rate tends to be a gradual process. It is, therefore, safer to increase the oxygen gradually during profound anesthesia.

ADMINISTRATION OF NITROUS OXIDE

Two systems are used for the induction of N_2O - O_2 anesthesia, namely, rapid induction and slow induction.

1. Rapid Induction

This mode of induction was advocated by E.I. McKesson (4). The patient is given a few breaths of pure nitrous oxide. As soon as the respiratory rate increases, about four or five percent oxygen is added. The oxygen is then gradually increased until the desired plane of anesthesia is reached. By treating the patient first with 100% nitrous oxide, the blood becomes saturated with the gas

more rapidly and less time is required to "saturate" the patient. This time may be reduced to a minimum if adequate premedication is administered (13). This type of induction is especially advised for patients who are resistant to narcosis or who have not been given premedication. Fast induction is very smooth (14), avoids the "excitement stage" and is more pleasant to the patient because he does not experience the unpleasant sensations which frequently occur when anesthesia is induced gradually.

2. Slow Induction

This type of induction is more advisable for children, subnormal patients or those classed as "poor risks". It consists of administering a mixture of 95/5 N_2O - O_2 until the patient looses consciousness, and then increasing the oxygen (13). This process is usually found to be unsatisfactory (14). Robust patients may struggle at first and may enter a stage of delirium. The time required to produce complete anesthesia is much increased.

Guedel (18) advocated slow induction of nitrous oxide anesthesia. He gave nitrous oxide with a high complement of oxygen for ten to fifteen minutes.

The technique of secondary saturation was introduced by McKesson (4). At any time during the operation the oxygen in the mixture is decreased in order to accelerate the production of the profound plane. Following this, oxygen is given to correct the overdosage and induce muscular re-

laxation. McKesson claimed that this method intensifies the anesthesia and produces sufficient relaxation for any type of operation.

The depth of narcosis is regulated by the proportion of nitrous oxide to oxygen in the continually administered gas mixture. The proportion of the gases is regulated to suit the patient and the duration of narcosis; the proportion of oxygen being increased in prolonged anesthesia. The commonly used mixture of gases contains 80% nitrous oxide and 20% oxygen (13).

Pre-oxygenation with 100% oxygen for 5 to 10 minutes before administration of nitrous oxide facilitates the induction of anesthesia. Neff et al. (14) state that the explanation of this fact lies

> "on the basis of an initial elimination of the body nitrogen, reducing the tension of this gas in the alveoli and allowing a corresponding increase in space for nitrous oxide."

It is not necessary to administer the gases under pressure (13). McKesson's practice is to administer the gas under slight pressure for the first few inhalations in the induction, but once the patient is unconscious the pressure is released.

Rebreathing means the re-inhalation of gases or air expelled during the preceding exhalation. Rebreathing may be partial or total. It maintains the carbon dioxide tension of the blood and stabilises the respiration. Partial rebreathing is more advantageous, because it eliminates

some of the carbon dioxide. In 1910, McKesson devised a method of partial rebreathing (4). Previously, the practice was to employ total rebreathing during anesthesia.

The rebreathing device is attached to the gassing apparatus and communicates with the mixing chamber. It may be set for any capacity up to 1,200 milliliters and acts as a trap to determine how much of each exhalation is allowed to escape. When the patient inhales, he takes in first that portion of the gas previously exhaled and then breathes in the mixture of N_2O-O_2. Rebreathing is used in anesthesia either to prevent the excessive loss of carbon dioxide due to overventilation, or to accumulate carbon dioxide when the respiration has been unduly depressed, prior to the operation, by excessive pre-anesthetic medication.

Rebreathing is not started until after the first few breaths, to avoid the prolongation of the induction period (4).

RECOVERY OF CONSCIOUSNESS

Recovery may be brought about by simply removing the mask and letting the patient breathe air, or by giving him a few breaths of pure oxygen (13). The latter method helps to avoid any "excitement reactions" when the patient comes out of the narcosis.

Nitrous oxide is displaced very rapidly by oxygen. The time required to regain consciousness varies with the patient and with the duration of anesthesia. Usually it is about one to three minutes.

APPARATUS USED IN NITROUS OXIDE ANESTHESIA

The development of various machines to improve and facilitate the administration of anesthetics has kept pace with the introduction of newer agents and methods.

Two methods gradually developed, the "continuous flow" method and the "intermittent flow" method (13). The continuous flow apparatus consists of flowmeters which indicate the rate of flow of each gas, the rate being regulated with valves. It is calibrated in liters or fractions of liters. The two gases are admitted by separate flowmeters into a supply bag. The proportions of the mixture can be regulated by varying the rate of flow. The patient inhales from the supply bag and exhales, usually only partially, into the atmosphere through an exhaling valve in the circuit.

The intermittent flow apparatus is automatic in operation. The mixing valve is provided with a graduated scale indicating the percentage of oxygen. During inspiration the velocity and volume of the gas mixture are automatically controlled by the rate and depth of the patient's respiratory efforts. During exhalation the flow of gases is automatically shut off. The mixture proportions in percentages are

determined by the movement of a single indicator. On increasing the proportion of one gas, the other is automatically decreased. The gas pressure may be adjusted by a regulator which controls the amount of gas rebreathed. An adjustable rebreather provides the control of rebreathing. This type of machine was perfected by McKesson.

An apparatus for carbon dioxide absorption may be attached to either apparatus mentioned. It consists essentially of a supply bag, a sodalime container for absorbing the carbon dioxide, and a flowmeter.

It is possible to administer oxygen alone if necessary in both types of apparatus.

PREMEDICATION AND DOSAGE

Premedication of the patient tends to intensify the action of the anesthetic (13). It lowers the metabolism and tends to retard the heart-rate, thus facilitating and accelerating the induction of narcosis. Gray and Cullen (19) stress the value of adequate or relatively high premedication in N_2O - O_2 anesthesia. Clement (13) states:

> "It is futile to attempt the use of N_2O - O_2 routinely for major surgery without particular care being given to the premedication of the patient."

The administration of the drug and the dosage should be regulated according to the age, sex, weight and the physical condition of the patient as well as the nature of the operation. Among the drugs most often used for premedication

are morphine, scopolamine, pentothal sodium and demerol.

Nitrous oxide is a very weak anesthetic and due to this it is not always possible to obtain narcosis with sufficient muscular relaxation necessary for certain operations. Previously this difficulty was overcome by inducing anesthesia with $N_2O - O_2$ and then substituting a stronger anesthetic agent such as cyclopropane or ether. Since the introduction of curare by H.R. Griffiths (20) into surgical use it has become possible to use this agent in $N_2O - O_2$ anesthesia to produce the desired degree of muscular relaxation. The use of $N_2O - O_2$ together with pentothal sodium and curare is now widespread. An 80/20 mixture of $N_2O - O_2$ produces anesthesia sufficient for operations not requiring a high degree of muscular relaxation (13). Analgesia may be produced, without loss of consciousness, with a 10/20 mixture of $N_2O - O_2$. The full effect is obtained in ten to twenty minutes (21). Seevers et al. (22) found that a gas mixture containing 35-40% nitrous oxide produced a maximum degree of analgesia, and yet the co-operation of the subject was retained.

ADVANTAGES AND DISADVANTAGES OF NITROUS OXIDE AS AN ANESTHETIC

The opinion of many anesthetists is voiced by Clement:

> "Nitrous oxide fulfils more nearly the requirements of the ideal anesthetic than any other general or inhalation agent in use at the present time." (13).

The following are among the advantages of nitrous oxide as an anesthetic:

1. Rapid absorption and ease of induction. Due to its great solubility in blood nitrous oxide is absorbed very rapidly and anesthesia is readily induced. Six or eight breaths of pure nitrous oxide are sufficient to bring about the loss of consciousness. This rapidity and ease of induction eliminates the displeasure to the patient.
2. Rapid elimination and recovery. Nitrous oxide is very quickly eliminated from the blood. Usually it takes from 1 - 5 minutes for the patient to recover fully. Thus the recovery is more rapid than with other commonly used anesthetics.
3. Absence of tissue change. Nitrous oxide has no irritant or toxic effect upon the heart, lung, kidneys or nervous tissues. It is the least toxic of all anesthetics as long as ample oxygen is administered with it.
4. Nausea. No nausea is experienced in 90% of the cases.
5. Non-inflammability. Being non-inflammable there is no danger of fire or explosion as with many other agents such as ether and cyclopropane.
6. Analgesia. Nitrous oxide is an ideal agent for the production of analgesia.
7. Safety. Nitrous oxide is the safest of anesthetics when administered by an experienced anesthetist, but can become very dangerous in unexperienced hands. The margin of anesthesia is very narrow and the depth of narco-

sis can be regulated as rapidly as desired. Overdosage with nitrous oxide develops very quickly but if the exposure is not prolonged the recovery occurs rapidly. The agent has no effect on the blood pressure.

There are also some disadvantages to the use of nitrous oxide (13).

1. Expense. The cost of the gases as well as of the equipment for their use is quite high.
2. Portability. The gases are supplied in tanks, which are cumbersome to transport. The apparatus is not conveniently portable.
3. Training and experience. Considerable training and experience in the use of N_2O - O_2 are essential for its safe and efficient administration.
4. Lack of potency. Nitrous oxide is a "light" anesthetic and does not always give complete muscular relaxation. This objection is among the most commonly cited. However, now that curare is used along with N_2O - O_2 anesthesia the objection is no longer a problem. Nitrous oxide plus curare fulfils the requirements for the perfect anesthetic (13).

THE MECHANISM OF THE ACTION OF NITROUS OXIDE

There are mainly two views as to the mechanism of action of nitrous oxide. Some authorities believe that hypoxemia

is an essential condition in the production of anesthesia, while others hold that nitrous oxide has a direct and specific action. The former view was commonly held at the beginning of this century, while the latter has received increasing support in recent years.

Leake and Hertzman (23) performed experiments on dogs using 90/10 and 85/15 mixtures of N_2O - O_2, and reported that it was impossible to produce true anesthesia without causing some degree of hypoxemia.

Brown et al. (24) also claimed that some degree of hypoxemia is an essential condition.

Clement (13) in his book on N_2O - O_2 anesthesia states that the reactions of the nervous system during anesthesia are typical of those obtained when the oxygen tension is reduced and are not due to the direct action of the gas itself. During the anesthesia the general body metabolism is decreased.

Faulconer and Pender (25), on the other hand, believe that:

> "Nitrous oxide acts specifically as an anesthetic agent and this action is possible in the presence of simple oxygen."

One of the very early explanations as to the action of nitrous oxide was given by B.W. Richardson (26). He believed that nitrous oxide was an asphyxiant, and that it produces its effects either by quickening the oxidation in the blood, which caused an accumulation of carbonic acid in

it; or by retarding the outward diffusion of carbonic acid.

The idea that nitrous oxide might influence the oxidation going on in the human organism arose quite early in the twentieth century. Wieland (27) claimed that the action of nitrous oxide is due to the interference with the oxidative processes of the central nervous system. Frankland and Coleman (28) carried out experiments from which they concluded that nitrous oxide when inhaled, undergoes little if any change and that it produces anesthesia by preventing oxidation. Gellhorn (29), on the other hand, expresses the view that

> "Anesthetics are usually distributed in all the cells and tissues and may influence their function independently of their action on the oxidative metabolism."

Adriani (30) also holds the view that nitrous oxide has no direct effect upon any of the body systems or tissues.

F.J. Prime (31) tried to determine the effects of nitrous oxide on the oxygen dissociation curve of whole blood. He found no significant shift in the curve in the presence of nitrous oxide. He used different carbon dioxide tensions, but did not determine the amount of nitrous oxide present in the gas mixtures. No chemical combination of nitrous oxide with blood hemoglobin has been demonstrated (16).

Gilmore *et al*. (32) found that a mixture containing 90% nitrous oxide and 10% oxygen caused a slight decrease in the coagulation time of the blood. However they found also that the time is significantly prolonged if the mixture contains

as little as 5% oxygen.

Bulow and Holmes (33), however, observed in *in vitro* studies that there was no inhibition of oxidation in guinea pig brain when no substrate was added, but if glucose was added as the substrate there was a 28% inhibition of the oxidative activity.

In a recent publication, Featherstone and Levy (35) report that they obtained no inhibition of brain respiration using glucose and pyruvate in the presence of nitrous oxide. They also studied the effect of nitrous oxide on oxidative phosphorylation and obtained no inhibition using brain homogenates and mitochondria.

Pittinger *et al.* (35a) have also observed no inhibition caused by nitrous oxide on the respiration of guinea pig brain hash with added glucose.

THE RESPIRATION OF ANIMAL TISSUE IN VITRO

The technique of measuring tissue respiration *in vitro* was developed by Warburg (36). He found that if a tissue from a freshly killed animal is either cut into thin slices or is homogenized and suspended in a physiological medium, it will continue to take up oxygen and give off carbon dioxide.

Brain tissue *in vitro* is dependent upon glucose or another suitable substrate and oxygen for the maintenance of respiration (37). According to Elliott *et al.* (38), lactate and glucose may serve as alternate substrates for brain. In the presence of glucose, the addition of lactate makes no significant difference in either the rate of respiration or of glycolysis. In the absence of glucose lactate can maintain the respiration at the normal rate.

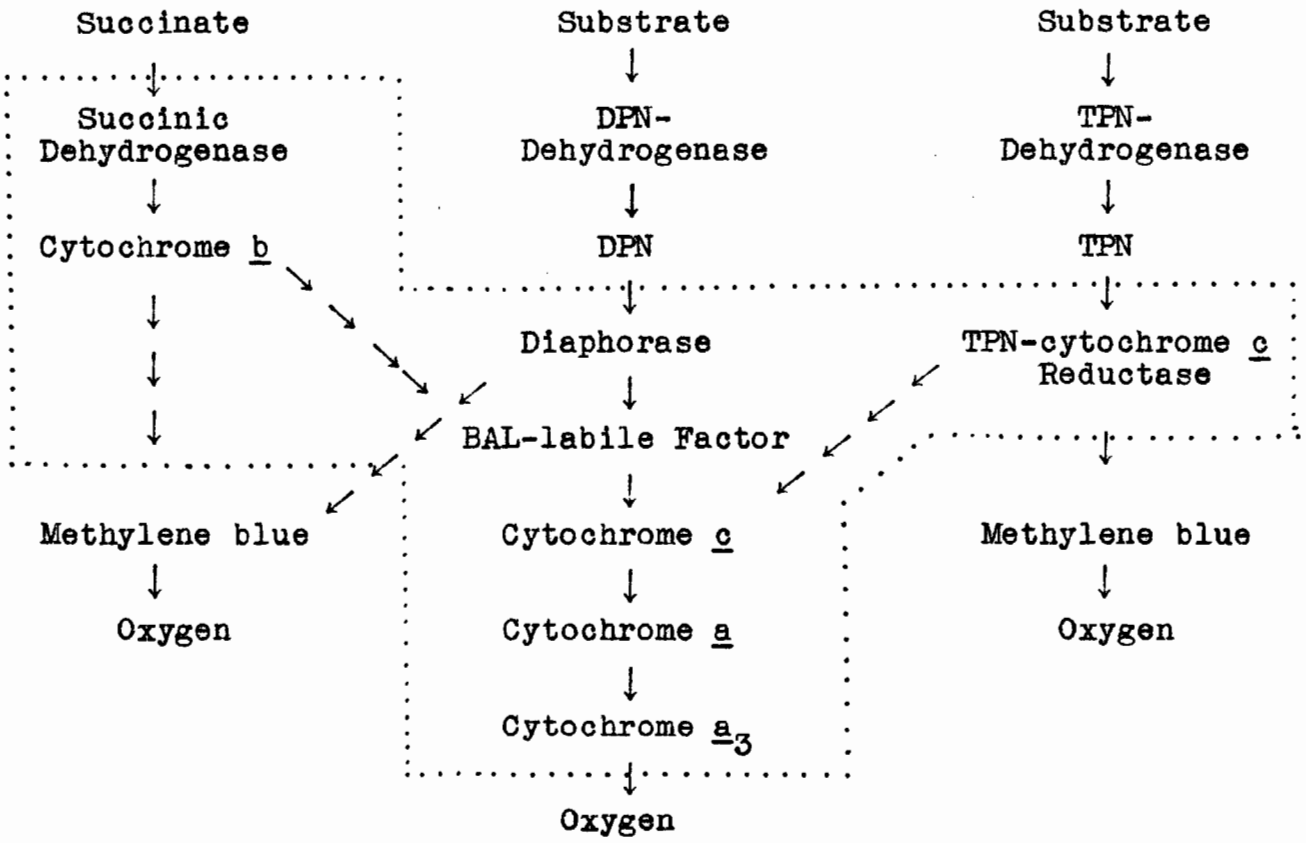
These workers also indicated that in rabbit kidney cortex, lactate is oxidized to pyruvate which, in turn, is oxidized in the tricarboxylic acid cycle. Lactate, added to liver slices, produces a slight increase in the rate of cellular respiration.

Succinate was found to be oxidized rapidly by brain, liver and kidney tissue (38 - 40). In every case the addition of succinate evoked a large increase in the oxygen consumption, but with brain the R.Q. tends to be slightly lower than with liver and kidney. With all these tissues the disappearance of the acid accounted for most of the oxygen utilized.

The addition of pyruvate to brain tissue increases its rate of oxygen uptake to the same extent as glucose (42). The

pyruvic acid disappears during this process (38, 43). The oxygen consumption of muscle, liver and kidney is increased by the addition of pyruvate to the same extent as by lactate (44).

THE RESPIRATORY CHAIN



The respiratory chain or electron-transport system is represented in the scheme above (45).

Components of the Succinoxidase System

In the scheme above, the components within the dotted

lines are all present in bound form (attached to insoluble submicroscopic particles) in standard heart-muscle succinoxidase preparations. Cytochrome c, diaphorase and TPN-cytochrome c reductase have been separated from the particles in soluble form and purified. Numerous claims to have brought cytochrome oxidase into solution and separated it into different factors have been discredited by Keilin and Hartree (46, 47). Most of such "purified" preparations have a lower Q_{O_2} than the starting material. These preparations could be reactivated by various methods, such as adding certain indifferent proteins.

It is considered that the catalysts in the colloidal particles, as in the intact cell, are more or less rigidly held together in a framework or mosaic which ensures their mutual accessibility. This would explain why endogenous cytochrome c in the preparations is reduced much more rapidly by succinate than is added cytochrome c (47), but it is reduced more slowly by chemical reducing agents such as ascorbic acid (48).

The disruption of the mosaic such as occurs during homogenization lowers the overall activity without necessarily destroying or removing any individual catalyst. Such a system can be reactivated by the addition of flocculent precipitates of foreign materials which provide new surfaces for reorientation.

Comparative studies by Slater (49) have shown that the succinoxidase system in kidney is essentially the same as that in heart-muscle.

Electron Transport from Succinate to Cytochrome c.

It is well known that cytochrome c constitutes a link between succinic dehydrogenase and the cytochrome-oxidase system. Important findings by Slater (50) showed that

1) Cytochrome b is an intermediary carrier between succinate and cytochrome c, and between succinate and dyes such as methylene blue;
2) a new respiratory catalyst inactivated by BAL (2:3 dimercaptopropanol) is an intermediary carrier between cytochrome b and cytochrome c, but not between cytochrome b and methylene blue. The identity of this BAL-labile factor is not known, but it has been suggested that it is a protein, with a prohaematin prosthetic group. It resembles haemoglobin in that it is easily destroyed by coupled oxidation with BAL. In the reduced form it does not show the absorption bands characteristic of haemochrome.

It has been suggested (51) that cytochrome b and succinic dehydrogenase are identical. In more recent work, however, the activity of succinic dehydrogenase has been separated from that of cytochrome b in a number of instances (52, 53).

Tsou (54) observed that succinic dehydrogenase could be slowly and irreversibly inhibited by cyanide in the absence of succinate. The enzyme can be protected from the cyanide by the addition of substrate or dithionite, but not by malonate or other competitive inhibitors of the enzyme, showing that it

is the oxidized form of the enzyme which combines with cyanide. When succinic dehydrogenase is almost completely inhibited, the cytochrome b is slowly reduced by the succinate, but it is eventually completely reduced showing that cytochrome b and the dehydrogenase are not identical.

Experiments on the kinetics of the reduction of the cytochromes have led Chance (55,56) to conclude that cytochrome b is reduced quite slowly and, therefore, does not participate in the main pathway of the electron-transport in the succinoxidase system. Slater (57) is not in agreement with Chance on this point.

In Slater's original scheme (50), succinic dehydrogenase was not placed in the main electron-transport pathway. However, Tsou (54) explained his observations that the cyanide inhibition of succinic dehydrogenase could be prevented by adding succinate or sodium hydrosulfite, by assuming that the cyanide reacts only with the oxidized form of the enzyme, which means that the enzyme itself is the immediate electron or hydrogen acceptor. The dehydrogenase was, consequently, considered to be a part of the main pathway.

Further evidence (58) for the existence of the BAL-labile factor between succinic dehydrogenase and cytochrome c is furnished by the inhibitory action of very low concentrations of antimycin-A on the succinoxidase system. They found that the inhibitory effect was not on the succinic dehydrogenase nor on the cytochrome c-cytochrome oxidase system; thus it seems to react with the postulated factor. Chance et al. (59)

have also shown that antimycin A inhibits respiration by combining with the intermediate factor. On the other hand, Widmer and Stotz (60) observed that antimycin A seemed to have its greatest effect on succinic dehydrogenase.

Clark *et al.* (52) have described a preparation of cytochrome *b* which has the same properties as the factor postulated to act between succinic dehydrogenase and cytochrome *c*.

Electron Transport between Coenzymes and Oxygen

The transport of electrons between reduced DPN and cytochrome *c* is not yet completely clear. Straub's diaphorase (61) oxidizes reduced DPN, but does not reduce cytochrome *c*. According to Slater (62) the diaphorase reacts with the cytochrome through an intermediary factor, namely the BAL-labile factor. He believes that the hypothetical "DPN-cytochrome *c* reductase" is made up of the diaphorase and the factor. In the process of purification of the diaphorase, the BAL-labile factor is eliminated thus resulting in the loss of the "reductase" activity.

Edelhoch *et al.* (63) and Mahler *et al.* (64) have isolated from pigeon breast muscle and heart muscle, respectively, flavoproteins which react directly with both reduced DPN and cytochrome *c*. These enzymes differ from the system involving the Keilin-Hartree heart-muscle preparation studied previously by Slater (49), in that they are not inhibited by BAL. Moreover, the prosthetic groups and the solubilities of the heart-

muscle enzymes are greatly different from those of Straub's flavoprotein (61). The preparations of Mahler *et al.* have diaphorase activity. Edelhoch *et al.* found that the ratio of cytochrome-reductase to diaphorase activity was increased with increasing concentration of alcohol and the temperature used during extraction.

Slater (57) has offered the following explanation to reconcile the above observations with his findings. He suggests that two systems are operative in the tissues for the oxidation of reduced DPN. One is a single enzyme such as that found by Mahler and Edelhoch, which reacts directly with cytochrome *c*. The second is the same system as that in the Keilin-Hartree muscle preparations. It, in turn, comprises two components, namely Straub's flavoprotein and the BAL-sensitive, antimycin-sensitive factor. Slater's postulate is supported by Potter and Reif's (65) finding of both the antimycin-sensitive and the antimycin-insensitive pathways for the oxidation of reduced DPN in heart muscle, liver and kidney homogenates. Antimycin completely inhibits the oxidation of reduced DPN by the Keilin-Hartree heart muscle preparation.

The electron-transfer route for TPN systems has been made fairly clear by the isolation from pig liver of TPN-cytochrome *c* reductase by Horecker (66). It is a flavoprotein which is reduced specifically by TPN; the reduced enzyme is then oxidized by cytochrome *c*.

Warburg (67), with improved measurements, showed that the turnover of cytochrome c in living yeast cells is sufficient to account for 95% of the total oxygen uptake. In other words, all the electron transport between the substrate and oxygen involves cytochrome c.

Electron Transport between Cytochrome c and Oxygen

Experiments described by Slater (49) support the view implied in the scheme on page 31 and confirm Keilin's observations on cytochrome a_3 and its CO-derivative and the probable identity of cytochrome a_3 with cytochrome oxidase. He has shown also that cytochrome b is not concerned in this part of the system.

Smith (68) has further purified the cytochrome oxidase preparations described by Smith and Stotz (69). Its absorption spectrum corresponds to that of cytochrome a and a_3. Study of the spectra after reduction indicates that cytochrome a_3 is related to, or identical with, cytochrome oxidase.

Ball et al. (70) have concluded from the study of the effect of carbon monoxide on the spectra of desoxycholate-dispersed heart-muscle preparations that cytochrome oxidase and cytochrome a_3 are identical. These observations are similar to those of Straub (71).

Intracellular Location of Respiratory Enzymes

The studies of Hogeboom et al. (72) on the distribution

of enzymes in the cell have led to the following findings:

1. The glycolytic enzymes occur almost entirely in the soluble form in the final supernatant liquid;
2. the main oxidation systems are concentrated in the mitochondria and to a lesser extent in the microsomes (submicroscopic particles);
3. the nuclei contain only about 5% of the oxidative enzymes.

Cytochrome oxidase, cytochrome c (73), and TPN-cytochrome c reductase (74) also have been found to be concentrated in the mitochondria which seem to be the main seat of the intracellular oxidations.

The Use of Artificial Hydrogen Acceptors in the Study of Tissue Metabolism

In the study of the enzymatic transport of hydrogen, several artificial hydrogen acceptors have been used. These acceptors terminate the series of reactions shown on page 31 at different stages. Among the most commonly used carriers are methylene blue and sodium ferricyanide.

(1) Methylene Blue.

Biological oxidation is the process whereby hydrogen is activated and removed from the metabolite. The dehydrogenases catalyze such reactions. Thunberg (75) was the first to study these reactions extensively by means of the decolorization of methylene blue. The activity of the system can be measured by the amount of time required to completely decolorize the dye under anaerobic conditions. It was found that the time requir-

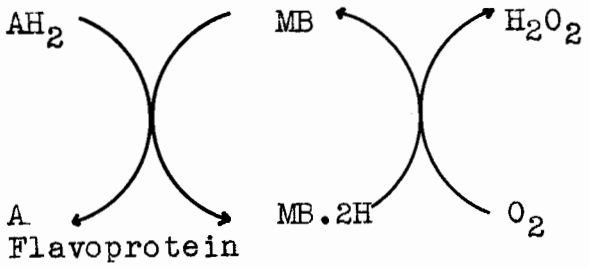
ed was inversely proportional to the amount of enzyme present, under standard experimental conditions. It is also possible to measure the activity of the system under aerobic conditions. In this case the amount of oxygen required to convert the leuco-dye to the original form is the index of activity. From his studies, Thunberg deduced that there are various kinds of dehydrogenases in living tissues.

Methylene blue was first used in comparative studies of normal and cancerous tissue by Fleisch (76). He was the first to show, that while cyanide inhibited the oxygen uptake of unwashed tissue respiring in the presence of various substrates, it did not effect the enzyme systems when the reaction was studied in the presence of methylene blue. From this he concluded that a "hydrogen transport factor" must be present in the tissues that can transfer hydrogen to methylene blue. Warburg and Christian (77) showed that their "yellow enzyme" was essential for the reduction of methylene blue.

It is now generally accepted that methylene blue interrupts the natural pathway of electron or hydrogen transport by accepting electrons or hydrogens from reduced flavoprotein. Hence, this artificial carrier by-passes the cytochrome-cytochrome oxidase system. Slater (50) showed that in the succinoxidase system, methylene blue accepts hydrogen or electrons from cytochrome b.

Baldwin has illustrated the role of methylene blue diagram-

matically as follows (78):



(2) Ferricyanide

The principal upon which the use of ferricyanide is based in manometric experiments was first described by Haas (79). When one mole of ferricyanide is reduced to ferrocyanide, one mole of hydrogen ion is liberated. If the reaction takes place in a bicarbonate medium, the liberation of the hydrogen ion causes the evolution of one mole of carbon dioxide which can be measured manometrically.

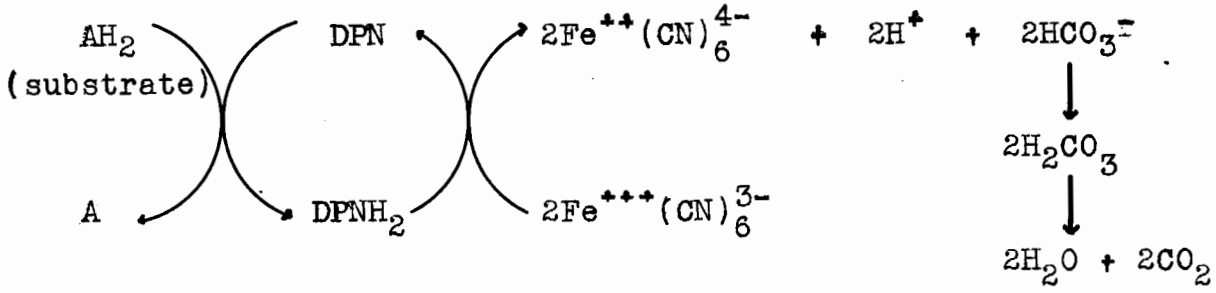
$$H + Fe^{+++}(CN)_6^{3-} \rightleftharpoons H^{+} + Fe^{++}(CN)_6^{4-}$$

$$H^{+} + HCO_3^{-} \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2\uparrow$$

Later, Mendel et al. (80) and other workers (81) found that 0.01M ferricyanide had little or no toxic effect on either respiration or anaerobic glycolysis in tissue.

The use of ferricyanide for the measurement of the dehydrogenase activity was introduced by Quastel and Wheatley

(82). These workers found that ferricyanide in concentrations used in manometric experiments is relatively non-toxic, and it has the advantage over most reduced dyestuffs of being non-autoxidizable. The overall reaction may be expressed thus:



This reagent does not require the intermediation of flavoprotein, but acts on reduced DPN directly, as was shown by Quastel and Wheatley (82). In systems not requiring DPN, as the succinoxidase system, ferricyanide probably acts directly on the dehydrogenase.

GLYCOLYSIS

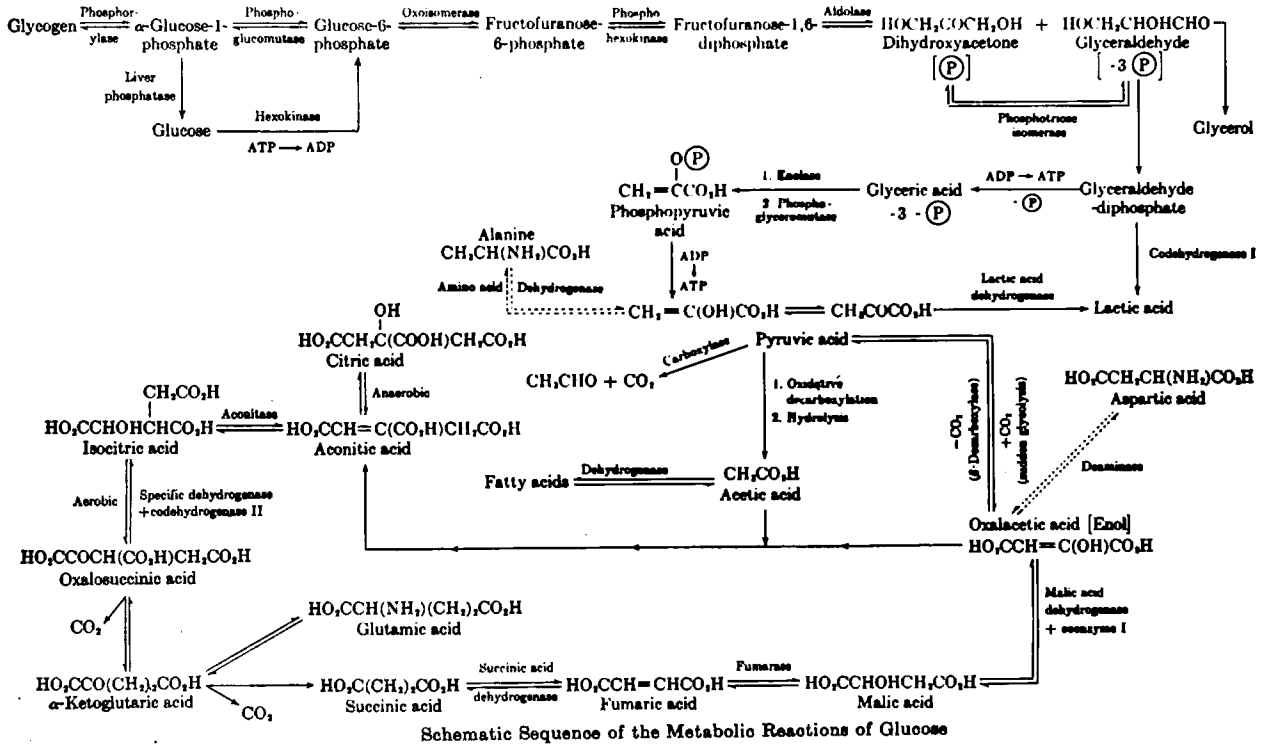
The glycolytic pathway of the degradation of carbohydrate is represented in the accompanying diagram. This scheme represents the combined contributions of numerous workers including Embden, Meyerhof, Parnas, Warburg, Cori, Dische, Hopkins and others.

Harden and Young (83) and later Robison (83) were the first to show the essential part played by phosphorylated sugars in alcoholic fermentation. Harden and Young also demonstrated

the presence of the coenzyme of fermentation, which they named cozymase. Its structure was elucidated later by Warburg and von Euler (83) and the compound was identified as diphosphonicotinamide-adenine dinucleotide, usually called diphosphopyridine nucleotide and abbreviated "DPN".

The anaerobic glycolytic system specifically requires DPN at the stages of oxidation by glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase.

The work of Meyerhof and his collaborators (84-86) has contributed a great deal to our present understanding of glycolysis. Their results clearly indicated the importance of adenosine triphosphate (ATP) in glycolysis and afforded an explanation of many previously disconnected and puzzling observations. The findings of these workers may be summarized as follows: Adenosine triphosphatase (ATP-ase) is bound to the structural elements of the cell and its activity is therefore higher in homogenates than in cell-free extracts. The concentration of ATP depends upon the ATP-ase activity and consequently tends to be higher in extracts and rapidly diminishes in homogenates. The phosphorylation of hexose and hexosemonophosphate requires a high concentration of ATP, and thus is slow in homogenates and rapid in extracts. The reverse is true for a substance such as hexose diphosphate (HDP) which is a phosphate donor. Its fermentation is slow in extracts but rapid in homogenates in which the continuous dephosphorylation of ATP maintains at a high rate the discharge



Schematic Sequence of the Metabolic Reactions of Glucose

Schematic Sequence of the Metabolic Reactions of Glucose (186)

Figure 1

of high energy phosphate from diphosphoglyceric and phosphopyruvic acids. The fermentation of HDP in extracts can be accelerated by adding purified ATP-ase or arsenate which breaks the coupling between the dephosphorylation and the ATP system.

A decrease in the ATP concentration lowers the affinity of brain hexokinase for fructose more than for glucose. Because of this, the fermentation of glucose is much more rapid than that of fructose in homogenates, but the rate is the same for both sugars in extracts. The difference between the fermentation rates of glucose and fructose in the homogenate can be abolished by the addition of ATP or other phosphate donors, or by lowering the activity of ATP-ase by means of inhibitors or by dilution.

More recent studies of Meyerhof et al. (87) have shown that the maintenance of an optimal ATP:ADP ratio is the most frequently encountered difficulty in glycolysis. These workers have shown that glycolysis may stop as a result of an imbalance between the rate of ATP breakdown compared with that of its synthesis. If the dephosphorylation of ATP is too slow, as with certain types of yeast-maceration juice, HDP tends to accumulate and fermentation ceases because of a lack of ADP or other phosphate acceptors. This effect can be eliminated by the addition of ATP-ase. However, if too much ATP is present, glucose phosphorylation ceases after a short period. In this case the addition of ATP-ase or hexokinase

helps to maintain the glycolytic activity. To obtain constant activity, the ATP-ase should be twice as active as the hexokinase, since twice as much ATP is synthesized as is used during glycolysis.

The comparison of inhibitors on the rate of glucose and HDP glycolysis has frequently been used as an indication of the activity of an inhibitor on hexokinase. On the basis of such studies Greig (88) has indicated that hexokinase is inhibited by amidone, and Meyerhof et al. (86) have shown that capryl alcohol and phenyl-urethane inhibit hexokinase and phosphohexokinase.

The transfer of phosphate from glucose-1-phosphate to glucose-6-phosphate has been explained in several ways. Schlamowitz and Greenberg (89) suggested that it was an intramolecular reaction, a diester monophosphate being formed which then split at position one to give glucose-6-phosphate. Leloir et al. (90) first proposed the formation of glucose-1,6-diphosphate as an intermediate; and later (91) isolated a new enzyme from yeast and rabbit muscle which they called glucose-1-phosphokinase. It catalyzes the reaction:

Glucose-1-phosphate + ATP $\rightarrow$ Glucose-1,6-diphosphate + ADP

Much supporting evidence (92, 93) has been presented to substantiate the occurrence of the following reaction:

Glucose-1,6-diphosphate + glucose-1-phosphate
$\rightleftharpoons$ Glucose-6-phosphate + glucose-1,6-diphosphate

Meyerhof and Oesper (94) studied the oxidative reaction of fermentation in detail and expressed it by the following equation:

D-glyceraldehyde-3-phosphate + phosphate + DPN^+ $\rightleftarrows$ 1,3-diphosphoglyceric acid + DPNH and H^+

The next step is the formation of 3-phosphoglyceric acid accompanied by the phosphorylation of ADP to ATP. The enzyme catalyzing this reaction has been purified and crystallized by Bucher (95). It requires magnesium ions.

Sutherland et al. (96) have shown that 3-phosphoglyceric acid is converted to 2-phosphoglyceric acid. The enzyme involved is phosphoglyceric mutase which is activated by 2,3-diphosphoglyceric acid present in catalytic amounts. Greenwald isolated this compound from red blood cells in 1925 (97).

Rapoport and Luebering (98) suggest the following sequence:

ATP + 3-phosphoglycerate $\rightleftarrows$ ADP + 1,3-diphosphoglycerate $\rightarrow$ 2,3-diphosphoglycerate

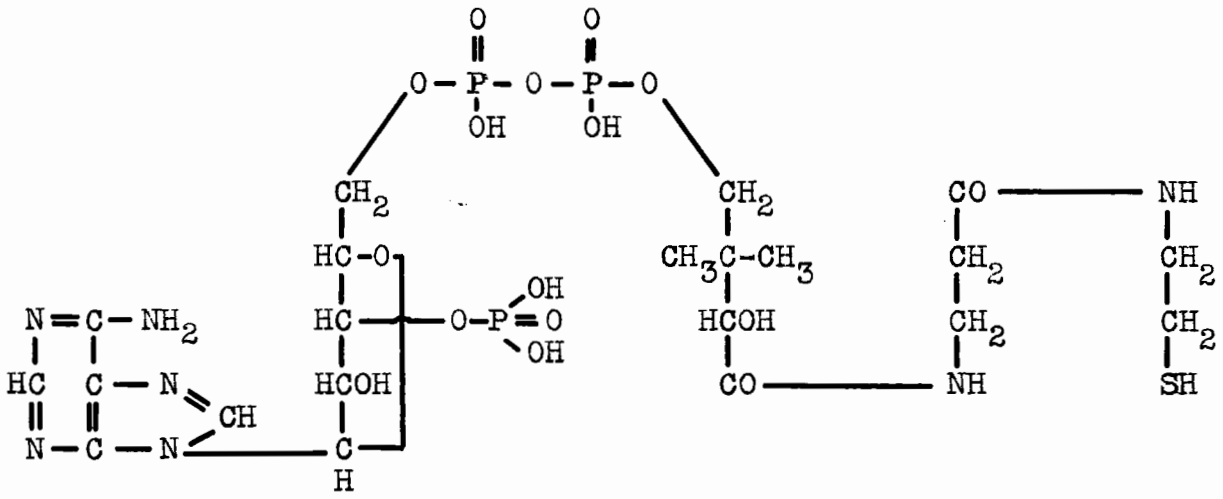
Two enzymes are required, one that of Bucher, the other, a new mutase which is found only in those red blood cells that contain 2,3-diphosphoglycerate. In subsequent work (99) they partially purified this enzyme and found that it required 3-phosphoglycerate as an activator.

Needham et al. (100) found that D-glyceraldehyde competitively inhibits the oxidation of D-glyceraldehyde-3-phosphate by triosephosphate dehydrogenase.

ACETYLATIONS

The mechanism by which acetylation is brought about in the organism has long engaged the attention of biochemists. Various hypotheses have been offered, but not until the discovery of Coenzyme A (CoA) in 1945 by Lipmann (101) has the elucidation of the problem been forthcoming.

CoA has been isolated in pure form. Its structure has been found to be the following (102):



The most reactive part of the CoA molecule is the sulfhydryl group (103).

Before the isolation of CoA, Lipmann (104) had discovered that a coenzyme was required not only for the acetylation of amines, but also for bringing about condensation reactions such as those involved in the synthesis of acetoacetate and citrate. Kaplan and Lipmann (105) showed also that the acetylation of sulfanilamide by extracts of acetone-dried pigeon liver required

acetate, ATP, and CoA. The requirement of ATP and CoA for the formation of citrate from acetate and acetoacetate was shown by Stern and Ochoa (106). The condensing enzyme which catalyzes the above reaction was isolated by Ochoa and his associates (107).

On the basis of these findings it was suggested that the "active acetate" involved in acetylations was acetyl-CoA. The actual proof of this, however, was given by Lynen et al. (108) who showed that a concentrate of acetyl-CoA which they prepared from yeast, could replace acetyl donor systems (acetate, ATP and CoA) required for the acetylation of sulfanilamide in the pigeon liver preparation described by Kaplan and Lipmann (105). The acetyl group of acetyl-CoA is combined with the sulfhydryl group (103) and thus forms a high-energy bond (109). Acetyl-CoA plays an intermediary role between acetyl donors and acetyl-acceptors (110).

Nachmansohn and Machado (111) were the first to show that in the reaction in which acetate was activated to an acetyl donor, ATP is required. It is now well established that ATP is necessary for the formation of acetyl-CoA. However, the mechanism of the formation of acetyl-CoA from acetate and ATP in yeast and animal tissue remains obscure. Lynen et al. (108) suggested the possibility of the formation of a phosphorylated form of CoA followed by the exchange of phosphate for acetate. This idea was given support by the experiments of Lipmann et al. (112) who formulated the following two-step oxidation:

(1) ATP + CoA $\rightleftarrows$ AMP + CoA-pyrophosphate

(2) CoA-pyrophosphate + acetate $\rightleftarrows$ acetyl-CoA + pyrophosphate

This reaction favors the formation of acetyl-CoA. Nachmansohn et al. (113) have prepared from pigeon liver an enzyme which catalyzes the overall reaction.

Lipmann (104) in 1948, showed that acetyl phosphate is not formed in animal tissues. It has been shown more recently that there is an enzyme, acetyl-CoA deacylase, which breaks acetyl-CoA into free acetate and CoA (114).

Acetyl-CoA + H_2O $\rightarrow$ acetic acid + CoA

A connection between CoA and the oxidation of pyruvate to acetate was established by the work of Dorfman et al. (115), Hills (116), Pilgrim et al. (117), Olsen and Kaplan (118), and by Littlefield and Sanadi (119). Later work (120) firmly established that the sequence of reactions in extracts of heart and pigeon breast muscle is as follows:

$CH_3COCOOH$ + CoA + DPN^+ $\longleftrightarrow$ Acetyl-CoA + DPNH + CO_2 + H^+

The CoA can be regenerated by either of the following ways:

(a) Acetyl-CoA + H_3PO_4 $\rightleftarrows$ acetyl-phosphate + CoA

(b) Acetyl-CoA + oxaloacetate $\rightleftarrows$ CoA + citrate

Reaction (a) is catalyzed only by phosphotransacetylase of

bacterial extracts while reaction (b) is catalyzed by the more widely distributed "condensing enzyme". Littlefield and Sanadi (119) succeeded in isolating acetyl-CoA as the product of the primary reaction.

The conversion of α-ketoglutarate to succinate has been studied in detail by Kaufman (121). It involves both DPN and CoA and is explained in the following reactions:

(a) α-ketoglutarate + DPN^+ + CoA → Succinyl-CoA + CO_2 + DPNH + H^+

(b) Succinyl-CoA + H_2O → Succinate + CoA

(c) Succinyl-CoA + ADP + P → Succinate + CoA + ATP

Kaufman (122) has suggested that the intermediate compound formed in step (c) is CoA-monophosphate, but there is no direct evidence for this. This step is catalyzed by the phosphorylating enzyme, the "P enzyme". Whether the latter and the dehydrogenase catalyzed step (a) are single entities remains an open question (123). The dehydrogenase has been purified (114, 124) and more recently, Littlefield and Sanadi (125) have purified and studied the "P enzyme".

Step (b) of the above reaction is catalyzed by succinyl-CoA deacylase which has been isolated by Gergely et al. (114). This reaction is analogous to the hydrolysis of acetyl-CoA.

The reactions involved in the formation of acetyl-CoA and succinyl-CoA respectively are not only catalyzed by different enzymes, but their mechanisms are different. In the acetate

reaction, acetyl-CoA, AMP and inorganic pyrophosphate are formed whereas in the succinate reaction, succinyl-CoA, ADP and inorganic orthophosphate are produced.

Choline Oxidase

Bernheim and Bernheim (126) showed that liver suspensions can oxidize choline. They found that acetylcholine is unaffected by this tissue preparation, but once hydrolyzed, the choline is readily oxidized. This implies that the free alcohol group of choline is necessary for the reaction. The rate of oxidation of choline depends on the pH of the reaction medium. At pH 7.2 the average amount of oxygen consumed was one and a half atoms per mole of choline. Trowell (127) showed that liver slices can also oxidize choline.

Bernheim and Bernheim (128) purified the enzyme and showed that it is distinct from the enzyme that catalyzes the oxidation of ethyl alcohol. From the measurement of the oxygen consumption, they concluded that when the reaction was carried out in a slightly acid medium, betaine aldehyde was formed, whereas in alkaline solution, betaine itself is the product. They were unable to isolate these products.

Mann et al. (129) confirmed the findings of Bernheim and Bernheim and succeeded in isolating and identifying the product of oxidation of choline as betaine aldehyde. They also obtained some evidence that this aldehyde is oxidized as well by liver tissue though much more slowly than choline, and that the product of the reaction is betaine. Mann and Quastel

(130) showed that one of the components of the choline-oxidizing system is a dehydrogenase, which can bring about the oxidation of choline anaerobically, provided a suitable hydrogen acceptor is present. The dehydrogenase is insensitive to low concentrations of cyanide, although the complete system is cyanide sensitive. These workers showed also that on incubating the dehydrogenase in the presence of choline or arsenocholine, under anaerobic conditions a rapid reduction of cytochrome c occurred. They postulated that hydrogen is transferred from choline dehydrogenase to the cytochrome-oxidase system. It was later observed by Williams et al. (131) that added cytochrome c has no effect on the rat liver enzyme. As DPN inhibited the reaction, they concluded that no additional cofactor was required for the system.

Recently, however, Strength et al. (132) have reported that a choline dehydrogenase obtained by the differential centrifugation of rat liver homogenate required DPN for optimal activity.

The dehydrogenase is inhibited by ammonium and trimethyl ammonium ions and by betaine, thus indicating that the nitrogen group and not the alcohol group of the substrate is of primary importance for the attachement of the enzyme (129). The enzyme is inhibited also by stearic acid (133), by the nitrogen mustard compounds (134), and by high oxygen tension (135).

Choline oxidase loses its activity very rapidly in washed liver homogenates (136) and cystine and semicarbazide in-

crease the rate of inactivation.

Williams (137) found that folic and ascorbic acids stimulate the choline oxidase activity of liver homogenates.

CARBONIC ANHYDRASE

In 1932, Meldrum and Roughton (138) isolated from ox blood a white crystalline substance which at a concentration of one part in ten million is active in catalysing the following reaction:

$$H_2CO_3 \rightleftarrows H_2O + CO_2$$

They called the enzyme carbonic anhydrase.

A mixture of bicarbonate and phosphate buffer at physiological pH will produce carbonic acid, from which CO_2 will be liberated. However, in the presence of carbonic anhydrase, the rate of decomposition of the carbonic acid is greatly increased. The rate of CO_2 evolution is, therefore, a measure of the activity of the enzyme.

Meldrum and Roughton found also that at low concentrations of enzyme, the acceleration of the reaction is directly proportional to the amount of the enzyme present. The enzyme is poisoned by carbon monoxide, cyanides, sulphides, azides, heavy metals, phenylurethane and sulphonamides (139-142).

Zinc has been found to be present in carbonic anhydrase and is necessary for the crystallization of the enzyme. Zinc appears not to be necessary for the activity of the enzyme, but there is some evidence that the inactivation of the enzyme by

inhibitors is due to a reaction between the inhibitor and the zinc (143).

The original method of estimating carbonic anhydrase was a manometric method developed by Meldrum and Roughton (139). Wilbur et al. (144) more recently have described an electrometric method. Krebs et al. (145) point out that the enzyme can be used to show that the carbon dioxide liberated in certain reactions, and not the bicarbonate anion, is the direct product of the reaction.

Van Goor (146) has published an excellent review of the extensive literature on carbonic anhydrase.

ACETYLCHOLINESTERASE

Loewi's original concept of the 'transmitter' action of the "Vagusstoff", which now has been identified as acetylcholine (Ach), implied the existence of some mechanism by which Ach, during activity or stimulation, is rapidly destroyed in the tissue. Subsequent studies (147, 148) confirmed that the destruction of acetylcholine is catalyzed by a widely distributed enzyme called "choline esterase" or "cholinesterase" (149).

There are two types of cholinesterases. One, called "pseudocholinesterase", is capable of hydrolysing several esters; the other, which is believed to be present only in brain, muscle and erythrocytes, is more specific for acetylcholine and thus is called "true" cholinesterase. Nachmansohn

et al.(150) have proposed the revised name"acetylcholinesterase" for this "true" cholinesterase, since this enzyme displays a high affinity for acetylcholine.

Loewi (151) was the first to demonstrate that the hydrolysis of Ach by tissue extracts is completely inhibited by eserine. The degree of inhibition depends on the temperature and the concentration of the inhibitor.

Diisopropyl fluorophosphate,tetraethyl pyrophosphate, prostigmine and eserine have an inhibitory effect on purified acetylcholinesterase (152). Tetraethyl pyrophosphate is among the most potent inhibitors known. The addition of acetylcholine and the inhibitor to the system affords some protection to the enzyme. This suggests that the inhibitors act on a common active center on the enzyme.

An excellent review on the enzymatic hydrolysis and synthesis of acetylcholine has been published by Nachmansohn and Wilson (153).

THE INHIBITION OF ENZYME SYSTEMS BY NARCOTICS

The work of Quastel and associates has elucidated the role played by drugs as enzyme inhibitors. Quastel and Wheatley (34) have shown further, that the power of various alkyl barbiturates to inhibit the oxygen consumption of guinea pig brain runs parallel to the hypnotic action of the particular drug. This parallelism is exhibited also among narcotics of various chemical types.

Chloretone and certain barbiturates, in concentrations that cause narcosis in animals, produce a definite inhibition of the respiration of cerebral cortex slices (154, 155).

The drugs do not act in the same manner on all the enzyme systems. The oxidation of glucose ,lactate and pyruvate is affected by chloretone and by a large number of other narcotic compounds. The oxidation of glutamate by brain slices is affected to a lesser extent by these drugs. None of the substances investigated, however, has any inhibitory action on the oxidation of succinate and p-phenylene diamine (156). The dehydrogenases involved in the oxidation of glucose, lactate, and pyruvate by brain also are insensitive to narcotics (157).

Anaerobic glycolysis of brain tissue is unaffected by narcotics in concentrations which markedly inhibit oxygen consumption (157) . It has been shown that aerobic glycolysis and glucose breakdown can be increased by various narcotics by suppression of the Pasteur Effect (158, 159) .

The lactic dehydrogenase of yeast and the glycerophosphate dehydrogenase of muscle are not inhibited by narcotics under anaerobic conditions, but the aerobic activity is markedly decreased(157). Jowett and Quastel (43) also studied the effect of such narcotics as luminal and evipan on the metabolism of liver, kidney and diaphragm. They found that the oxidation of lactate and glucose by these tissues was narcotic-sensitive.

The oxidation of succinate by liver was unaffected in the presence of luminal.

Watts (160) found that methadon, an analgesic drug, above certain concentration inhibits the oxidation of ascorbate, succinate, and glucose, but not anaerobic glycolysis.

Analysis of the various links in the respiratory chain leading to the oxidation of the substrate has revealed that one of the components of the multi-enzyme system is highly sensitive to narcotics (157, 161). Since the oxidation of succinate and p-phenylenediamine is not inhibited by narcotics, it was inferred that these substances have no effect on the cytochrome oxidase system, as these reactions depend on the utilization of molecular oxygen.

Therefore, since lactic and succinic dehydrogenases and the cytochrome system are not affected by narcotics, it was concluded that the narcotic-sensitive factor in the respiratory chain is situated somewhere between reduced DPN and the cytochromes in the reaction sequence.

Further studies led Michaelis and Quastel (157) to conclude that either the sensitive step is located between the flavoprotein and the cytochromes, or that a special protein that links the reduced DPN and the cytochrome system is narcotic-sensitive. From similar experiments Greig (161) suggested that the site of action of the narcotics is not at the DPN-flavoprotein coupling, since no accumulation of reduced DPN could be detected. Further, since lactic dehydrogenase of yeast is inhibited by narcotics under aerobic but not under anaerobic

conditions in the presence of methylene blue, Greig concluded that the interference of the narcotics is at some point between flavoproteins and cytochromes in the metabolic sequence.

After the discovery by Slater (49, 50) of an intermediary factor between diaphorase and cytochrome c, the possibility that it might be the narcotic-sensitive step was surmised. The inhibition of respiration by narcotics resembles that brought about by reducing agents, thus indicating that the factor may be sensitive. It is not yet certain whether narcotics act specifically on this factor or inactivate the respiratory system by a non-specific physical action on the cytoplasmic particles which contain the components of the respiratory chain (162).

Quastel and Wheatley (163) have shown that the inhibition by narcotics such as barbiturates and chloretone, is reversible. However, after exposure to high concentrations of drugs the inhibition can not be reversed.

Recent evidence obtained by Johnson and Quastel (164) leads to the conclusion that certain narcotics, such as chlorotone and nembutal at low concentrations inhibit an oxidative step in the cell respiration which is concerned with the synthesis of ATP, and thereby inhibit biological acetylations, such as those of choline or sulfanilamide. The inhibition of acetylation can be relieved by the addition of ATP to the aerobic system. Even high concentrations of the drugs do not inhibit the anaerobic synthesis of acetylcholine.

Eiler and McEwen (165) have shown that pentobarbital inhibits the generation of high-energy phosphate bonds and thus interferes with oxygen utilization. Brody and Bain (166) have postulated that an uncoupling of phosphorylation from oxidation may be connected with the narcotic action of barbiturates.

PREPARATIONS

Media for the Suspension of Tissue Preparations

Numerous suspension media were employed throughout this study. The Krebs-Ringer medium was used for both aerobic and anaerobic studies. Special media used for certain experiments will be described in the text.

The Krebs-Ringer solution used for aerobic studies comprised the following substances:

100 parts of 0.154 M NaCl
4 " " 0.154 M KCl
1 " " 0.154 M KH_2PO_4
1 " " 0.154 M $MgSO_4.7H_2O$
12 " " 0.1 M phosphate buffer (pH 7.4)
0.5 " " 2.0 M glucose

This solution will be referred to as Krebs-Ringer-Phosphate-Glucose (KRPG).

For the anaerobic studies, the medium described above was modified as follows: 21 parts of 0.154 M $NaHCO_3$ was substituted for the phosphate buffer and the solution was equilibrated with CO_2. Three parts of 0.11 M $CaCl_2$ were also added to this anaerobic medium. The $CaCl_2$ was omitted from the aerobic medium, since calcium tends to be precipitated in the presence of phosphate. The anaerobic medium will be referred to as KRBG.

All the solutions described above are isotonic and can,

therefore, be mixed in any proportions to yield a final isotonic solution.

Role of Nicotinamide in the Respiration of Homogenates

It was shown by Mann and Quastel (167) that nicotinamide in concentration of 1 or 2 x 10^{-2} M, prevents the breakdown of DPN by an enzyme which they called "cozymase nucleotidase". These findings were confirmed by Handler and Klein (168) who proved further that "cozymase nucleotidase" splits DPN at the junction of the nicotinamide and ribose. They concurred with Mann and Quastel's original suggestion that nicotinamide produces its effect by competing with DPN for the enzyme.

The "cozymase nucleotidase" of Mann and Quastel is commonly called DPN-ase. It is now known that there is another enzyme which splits DPN at the junction of the nicotinamide mononucleotide and adenylic acid components. This enzyme is not inhibited by nicotinamide (169).

Recent studies by Alivisatos (170) and others (171) have shown that DPN-linked dehydrogenases may be inhibited competitively by high concentrations of nicotinamide.

McIlwain and Rodnight (172) showed that the presence of 0.001 M nicotinamide effectively reduces the rate of breakdown of DPN when added to brain tissue.

In the experimental work described in the following sections, all the homogenates were prepared in media containing 0.017 M nicotinamide. This prevented the breakdown of

endogenous DPN and the homogenate could be used without further addition of DPN. This concentration of nicotinamide was found by Hochester (173) to be adequate for preserving the endogenous DPN of yeast preparations.

Tissue Preparations

Healthy albino rats were used as the source of the tissue preparation in our experiments. The animals were kindly supplied by the Charles E. Frosst & Co. Ltd., of Montreal. In an effort to minimize the influence of individual variation especially noticeable with liver tissue, all of the animals were kept on a purina diet.

The animals were killed by instantaneous decapitation by means of a specially constructed "guillotine". This method of sacrificing the animals made possible the rapid removal of the tissues. The brain, for example, could be removed in less than one minute.

(a) Tissue Slices

In the preparation of slices from brain, kidney or liver, the tissue was cut in a humidified cabinet with the aid of a Stadie-Riggs microtome (174). The slices were weighed immediately on a precision balance and promptly placed in the cold suspension medium in the Warburg vessels.

Slight variations tend to occur in the wet weight of tissues, even when the tissue is cut in a humid atmosphere. The loss of moisture, however, is much less than when the tissues are cut with a wetted blade or when they are stored

in a suitable volume of Ringer-type medium prior to being sliced.

The dry weight of the tissue slices was calculated from the commonly accepted ratio $\frac{\text{Wet weight}}{\text{Dry weight}}$. The ratio for the three tissues is: brain-5.0; liver-3.7; kidney-4.0 (175).

(b) Homogenates

The relation between the metabolism of homogenates and of tissue slices or extracts has been widely discussed in the literature. Potter (176) and Meyerhof (85) have found that the nature of the activity of the homogenate is determined by its mode of preparation. These workers have shown that the metabolic activity of the homogenates is comparable to that of slices, if the homogenization is carried out under certain conditions of speed, duration and temperature and with certain kinds and concentrations of suspension media. The results of experiments of Elliott and Henry (177) indicate that by their methods of preparing brain homogenates, the brain cells remain intact and the coenzymes remain attached to the structural elements. If the tissue is homogenized in distilled water, after the method of Utter et al. (178), the coenzymes are released and readily destroyed by the nucleotidases.

In this study, the methods of preparing the homogenates varied slightly according to the enzyme system to be studied. When the enzyme activity of the homogenate was to be compared directly to that of the tissue slices, the same suspension

medium was used for both preparations. Nicotinamide was added to the medium whenever the preservation of the endogenous DPN was essential. The piece of tissue to be homogenized was weighed and placed in the Potter- Elvehjem glass homogenizer containing an appropriate volume of the medium. In making the adjustment of the volume after homogenization, a volume-weight relationship of 1 ml./gm. of tissue was assumed and medium was added to give a final concentration of 100 mgs. of tissue/ml.

After homogenizing the tissue at a constant speed and for a definite period of time, portions of the cold, uniformly suspended homogenate were pipetted into the cold medium in the Warburg vessels.

(c) Pigeon Liver Acetone Powder

The pigeons were decapitated and the livers were immediately removed. They were homogenized for 60 seconds in the Waring blendor. Ice-cold acetone was added to facilitate the homogenization.

The homogenate was then added to 10 volumes of ice-cold acetone. After 5 minutes, the dehydrated material was filtered through a Buchner funnel and washed with acetone. The drying was completed in a vacuum desiccator over $CaCl_2$. The powder could be kept for more than a month at $0^{o}C$. in the vacuum desiccator without loss of activity.

(d) Pigeon Liver Extract

The aceton-dried powder was ground in a mortar in an aqueous medium. The medium used will be described in the text. The proportion of tissue to extraction medium was 120 mgs. of powder per ml. A small quantity of medium was first added to the powder to make a thick paste, and then the remaining fluid was added gradually with continuous grinding, the whole operation taking 5 minutes.

The suspension was centrifuged in the cold at 20,000 r.p.m. for five minutes. A volume of clear supernatant equivalent to 60 mgs. of the original dry powder, was pipetted into the prepared Warburg vessels.

Preparation of Substrates

The substrates were prepared in suitable buffer mixtures and the final pH was adjusted to that of the suspension medium used.

Some of the substrates were obtained in the form of the barium salts. These were dissolved in acid, an amount of Na_2SO_4 calculated to remove all of the Ba was added, and the precipitated $BaSO_4$ was removed. The clear solution was then neutralized with NaOH and made up to the desired volume with buffer.

METHODS

The majority of the experiments reported in this study

were performed with the Warburg apparatus. The temperature of the bath was kept constant at 37.5°C. except where otherwise indicated. The vessels were shaken at 135 oscillations per minute.

The gassing of the vessels was usually carried out with the aid of a flow-meter at a standard rate and for five to ten minutes prior to attaching the manometers to the Warburg apparatus. In the anaerobic experiments, one set of vessels was gassed with a gas mixture of 5% CO_2 and 95% N_2, freed from oxygen by passing it through a heated quartz tube containing copper turnings. The experimental vessels, in which the effect of N_2O was studied, were gassed in a similar manner with a gas mixture containing 5% CO_2 and 95% N_2O.

In the aerobic studies, air was used for gassing one set of vessels and a mixture of 80% N_2O and 20% O_2 was used in the experimental vessels.

The gas mixtures containing N_2O and either O_2 or CO_2 were prepared by displacing a certain volume of water by the appropriate gas from a 20 liter bottle. A known volume of N_2O was introduced into the bottle first, followed by a calculated volume of oxygen or carbon dioxide to produce the desired mixtures, allowances being made for the solubility of these gases in water. The mixture thus made was forced through the manometers by displacement with water previously saturated with N_2O.

The temperature equilibration period was usually ten minutes.

With aerobic experiments, the addition of the side-bulb contents was always at the start of the experiments (zero time). In the anaerobic experiments, the addition was made usually 10 minutes before zero time in the case of slices and homogenates, or three minutes with extracts. This interval was necessary to permit equilibration after mixing, even though all the ingredients were made up in an appropriate buffer.

The vessels used in the aerobic experiments were provided with 0.2 ml. of 20% KOH and a folded filter paper (35 $mm.^2$) in the center well of the Warburg vessel. When methylene blue was used, 0.2 ml. of 10^{-5} M solution of the dye was placed in the side bulb.

In the anaerobic studies, the ferricyanide method of Quastel and Wheatley (179) was used. The concentration of ferricyanide used (2.5×10^{-2} M) was considered non-toxic by these authors.

Determination of Sulfanilamide

Sulfanilamide was determined according to the method of Bratton and Marshall (180). The principle of the method is as follows: Sulfanilamide is diazotized with nitrous acid, ammonium sulfamate is added to destroy the excess nitrous acid, and the diazotized sulfanilamide is coupled with a color reagent, N-(1-naphthyl)-ethylenediamine dihydrochloride. A red color is produced very rapidly and is uninfluenced by a strong-

ly acidic medium (less than pH 2). The maximum absorption using the Evelyn photoelectric colorimeter is at 540 mu. This method is very sensitive. It measures from 1 to 10 ug sulfanilamide.

Trichloroacetic acid (TCA) was used as the protein precipitant. It gives a negligible blank value with the reagent.

The analysis of the contents of the Warburg vessel was performed by the following procedure: The contents of the flask (3 ml.) were precipitated with 3 ml. of 3.2% TCA. The precipitate was centrifuged at 2,500 r.p.m. for 8 minutes in the cold, and 2 ml. of the supernatant were diluted to 50 ml. with 3.2% TCA. The rest of the determination was done on a 10 ml. sample of the diluted supernatant. One ml. of 100 mg. % $NaNO_2$ was added. After 3 minutes, 1 ml. of 500 mg.% sulfamate was added. After 2 minutes, 1 ml. of the color reagent was added and the optical density of the color was read immediately in the colorimeter at 540 mμ.

EXPERIMENTAL

PRELIMINARY EXPERIMENTS

The use of nitrous oxide as an anesthetic is widespread. It produces anesthesia rapidly and the recovery of the patient occurs promptly when the mask is removed.

Most of the experiments presented in this thesis were performed on rat tissue *in vitro*. Preliminary to starting these experiments, however, it was of interest to see if N_2O produced an effect on rats *in vivo*, similar to that exerted on human patients.

The experiments were performed in the following manner. Male rats of uniform weight were used. In each experiment one animal was placed in a bell jar through which a mixture of N_2O-O_2 was then passed. Different mixtures of gases were used and each animal was exposed to the gas for a different period of time.

In the first set of experiments the rat was placed in the bell jar and a 2:1 mixture of N_2O-O_2 was passed through for 5 minutes. The gas supply was then shut off and the animal left in that atmosphere for a definite period of time, the duration differing with each experiment. Before the onset of anesthesia, the rat tended to struggle a little, but this lasted only a few moments. By the end of two or three minutes the animal had usually lost consciousness and was deeply anesthetized. It could be kept under this condition for

periods up to 55 minutes without showing any sign of asphyxia. The breathing remained regular throughout. However, if the rat was not taken out of the bell jar at this time, its breathing usually became erratic and convulsive movements were evident. If the bell jar was now removed the rat would make a complete recovery within 2 or 3 minutes.

The gasping and convulsions which usually occur at the end of the hour are probably caused by the lack of oxygen and not by the direct action of the nitrous oxide itself. The rat thus kept in a closed space used up the oxygen and produced CO_2 which saturated the atmosphere.

To prove that anoxia was responsible for these effects, experiments were done during which the 2:1 mixture of N_2O:O_2 was passed through the bell jar continuously. In this manner the animal could be kept anesthetized for periods up to several hours. The breathing remained normal, regardless of the length of the period. When the bell jar was removed, the rat regained consciousness within 2 or 3 minutes.

The next set of experiments was done using a 4:1 mixture of N_2O and O_2. The gases were passed through the bell jar for 5 minutes and the supply was then shut off. The rat can remain in such an atmosphere for 30 minutes , after which time it tends to show signs of asphyxia probably because all the oxygen has been used up. Again the recovery is very rapid, and within 2 or 3 minutes the rat behaves in a normal manner.

These experiments show that N_2O has the same anesthetic effect in the rat as in the human, and that recovery also is as rapid and uneventful as in man.

The Effect of Nitrous Oxide on Blood.

The following experiments were performed to see if N_2O interferes with the functioning of oxyhemoglobin.

Fresh blood from the rat was collected in 3.2% citrate and diluted 1:50 with distilled water. Pure nitrous oxide was bubbled through 5 ml.samples of this diluted blood for different lengths of time.

After the gas had been bubbled through the solution for two or three minutes, the color of the blood changed from a bright red to a dark rose. The blood sample was then examined in a Hartridge spectroscope. It was found that oxyhemoglobin bands had disappeared and were replaced by a single wide band of reduced hemoglobin. The change was usually complete within two or three minutes since no further change could be detected when the sample was gassed for five, ten, fifteen or twenty minutes.

The gassed specimen was allowed to stand in an open test tube. After about a minute or two the bands of oxyhemoglobin began to appear and they became darker on standing. The maximum time required to recover the bands of oxyhemoglobin was fifteen minutes. The time required for this

recovery was directly proportional to the duration of the previous exposure of the sample to the nitrous oxide.

Concurrently with the reappearance of the oxyhemoglobin bands the sample resumed its characteristic bright red color.

When pure oxygen was bubbled through blood that had been treated with N_2O, the oxyhemoglobin bands and the bright red color would reappear almost immediately.

The disappearance of the oxyhemoglobin bands on exposure of blood to N_2O is accompanied by a slight change in the pH of the sample. The pH before gassing was 7.28, and after bubbling N_2O through it for four minutes was 7.5. If the specimen was exposed to the air for fifteen minutes, the pH returned to 7.25.

The effects described above can be obtained only by bubbling pure N_2O through the blood. A mixture of 80% N_2O: 20% O_2, bubbled for 45 minutes, produced no change in the color of the blood nor in the oxyhemoglobin absorption spectrum.

These experiments indicate that pure nitrous oxide tends to expel oxygen from the blood and from oxyhemoglobin. Whether the N_2O replaces the oxygen by combining itself with the hemoglobin is not known and seems rather unlikely, since the product according to the absorption spectrum is simply reduced hemoglobin. If any complex is formed, it must have an absorption optimum like that of hemoglobin and must be extremely unstable, for it is very readily broken down when

oxygen is again readmitted to the system.

Since bubbling an 80:20 mixture of N_2O:O_2 through blood specimens produces no visible changes, it is safe to say that this mixture, which is the one most commonly used in anesthesia, causes no alteration in the oxyhemoglobin in the body. Long experience with nitrous oxide in clinical anesthesia has confirmed this beyond doubt.

CALCULATION OF PERCENTAGE INHIBITION

In estimating the effect of various agents on cellular metabolism, the most satisfactory way of presenting the results is in the form of percentage inhibition.

The method of calculating the percentage inhibition can be easily shown by the following example:

Experiment

The Effect of Nitrous Oxide on the Respiration of Rat Brain Cortex Slices

System	Air	80% N_2O:20 % O_2
Control	(7.8)*	(3.7)*
" + Lactate 0.01 M	11.7	5.8

Experimental conditions as in Appendix i , P. 150

*NOTE: ( ) The parentheses have been used in the Appendices to designate the control, which is the complete system without substrate.

In the presence of lactate the $-Q_{O_2}$ of brain slices was found to be 11.7. With N_2O:O_2 as the gas phase, the $-Q_{O_2}$ was 5.8, indicating a marked inhibition of the respiration. Assuming the $-Q_{O_2}$ of 11.7 as 100% activity, the $-Q_{O_2}$ of 5.8 represents an inhibition of 50%.

THE EFFECT OF NITROUS OXIDE ON THE TRANSPORT OF HYDROGEN.

BRAIN METABOLISM

The Effect of Nitrous Oxide on Brain Metabolism

The theories which have been offered to explain the phenomenon of narcosis have already been referred to in the introduction of this thesis. The tissue oxidation theory, elucidated by Quastel and his colleagues, explains the narcotic effect of drugs by the fact that they produce an inhibitory effect on the metabolism of brain tissue. By the term "narcotic", these workers include all drugs as well as gaseous anesthetics. In their investigation of the action of N_2O, Quastel and Wheatley (34) found that the oxygen consumption of brain tissue was decreased by 28% in the presence of glucose, but remained unchanged when no substrate was added. They concluded, however, that the degree of inhibition had been underestimated in view of the high solubility of N_2O in the medium. These results were disputed by Bulow (181) who found no inhibition of respiration of chopped brain under similar conditions. Bulow's view is upheld by Pittinger *et al.* (35a) and by Levy and Featherstone (35) who have been unable to show any inhibition of brain tissue, respiring in the presence of glucose and pyruvate. These workers, therefore, suggest that N_2O should not be classed as a narcotic, since it does not conform with the oxidation theory of narcosis.

In view of these contradictory opinions, we considered it advisable to study the effect of nitrous oxide on various enzyme systems in brain tissue in the presence of various substrates.

The most commonly used gas mixture for anesthesia is 80% N_2O-20% O_2. This proportion of the gases was used in all the aerobic experiments. The inhibitory effect could not be attributed to a low oxygen tension, since the controls were respiring in air.

The results of the experiments are presented in Table I.

(a) The Succinoxidase System

The pathway of succinate oxidation has already been discussed on page 31. In the experiments described below, succinate was added to brain slices or to homogenates respiring in a Krebs-Ringer-Phosphate-Glucose medium. The addition of succinate produced a considerable increase in the oxygen consumption of the tissue.

The oxygen consumption of brain slices respiring in an N_2O-O_2 atmosphere, was about one half of those respiring in air. When the brain tissue was in the form of a homogenate, however, the inhibition was very much lower, only 18%.

(b) DPN-linked Systems

A number of the known dehydrogenases specifically require

DPN as a coenzyme for hydrogen or electron transport. A few of these systems have been studied in the present investigation.

Table 1

The Effect of Nitrous Oxide on the Respiration of Rat Brain Slices and Homogenates (Medium KRPG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	53	10
Succinate	49	18
Lactate	50	22
Pyruvate	65	36
Iso-citrate	59	16

For values see Appendices i and ii

(i) No Added Substrate.

The controls respire in a KRPG medium without the addition of extra substrate. Some of the glucose present in the medium can be oxidized to gluconic acid by glucose dehydrogenase. This enzyme can use both DPN or TPN as a coenzyme, but has a

higher affinity for DPN.

The oxygen consumption of the slices without added substrate is inhibited by 53% in the presence of N_2O. The respiration of the homogenate under the same conditions is inhibited by only 10%.

(ii) Lactate

l-lactic acid is oxidized in the tissue to pyruvic acid through the lactic dehydrogenase system. This system requires DPN specifically for its activity. The addition of lactate to brain slices and homogenates produces a very small increase in the oxygen consumption. In an N_2O-O_2 atmosphere, the oxygen consumption was decreased by 50% in the case of brain slices, and the respiration of the homogenate was decreased by 22%, in the presence of lactate.

(iii) Pyruvate

The addition of pyruvic acid to the medium did not greatly alter the oxygen consumption. The pyruvate oxidizing system also requires DPN.

The metabolism of pyruvate by brain slices was inhibited by N_2O to an extent of 65%. The degree of inhibition was somewhat greater than in the systems previously noted. The metabolism of pyruvate by brain homogenate was decreased by only 36% in the presence of N_2O.

(c) TPN-linked Systems

Certain dehydrogenases require TPN as the coenzyme in the electron transport system. The effect of N_2O on one of these enzymes was studied.

(i) Iso-citrate

The addition of iso-citrate does not greatly alter the respiratory activity of brain slices. Nitrous oxide inhibited this system by 59%. A negligible degree of inhibition (16%) was obtained with brain homogenates.

DISCUSSION

It is evident from the results of the experiments just described that the oxygen consumption of brain tissue respiring in an atmosphere of 80% N_2O and 20% O_2, is decreased. The degree of inhibition varies greatly with slices and homogenates.

With brain slices the addition of various substrates influences slightly the degree of inhibition. The greatest inhibition, 65% was obtained when pyruvate was used as the substrate. The range of inhibition for the other substrates, succinate, lactate and iso-citrate, varied between 49% and 59%.

With homogenates, on the other hand, the degree of inhibition tends to be much lower, ranging from 10% to 36%. This marked difference between the behaviour of slices and

homogenates implies that there must be in slices a N_2O sensitive component of the hydrogen transport system which is either destroyed or by-passed in the homogenate. It is unlikely that homogenization should destroy any of the components of the system. However, disintegration of the tissue may disturb the natural organization of the cell without destroying any enzymes, and thus in some manner alter the sequence of reactions described on page 31.

The previous studies of Bulow (33), of Pittinger et al. (35a), and of Levy and Featherstone (35) have indicated that the respiration of brain tissue is not inhibited by the presence of N_2O. Bulow used guinea pig brain minces respiring in the presence of glucose. Our results in Table I show that rat brain homogenates, without added substrate, were inhibited only to the extent of 10%. This low inhibition is nearly within the margin of experimental error of the method and thus is of doubtful significance. It is, therefore, reasonable to say that the results obtained with brain homogenates are in agreement with those of Bulow. Since the inhibition with slices was so much higher than in homogenates, it cannot be compared with Bulow's results.

Quastel and Wheatley (34) used "whole guinea pig brain". They obtained no inhibition without added substrate, but a 28% inhibition in the presence of glucose. In our experiments, the medium for the controls without substrate contained 0.007 M glucose, and therefore our controls are comparable with

Quastel's controls containing glucose. The 28% inhibition obtained by Quastel is lower than our values with slices, but higher than that obtained with the homogenates. The diffusion of the gas throughout the tissue may possibly account for this difference in results. The slices used in our experiments were of a uniform thickness (0.5 mm.). The nitrous oxide could easily penetrate all the cells of the slice and thus alter the respiratory activity of the whole slice. Quastel used pieces of whole brain which were probably several millimeters thick, and under such conditions the N_2O might not diffuse uniformly throughout all the cells. The inhibition of respiration, therefore, might be variable throughout the tissue depending on the diffusion of the gas.

Pittinger et al. (35a) used guinea pig brain hash, and Levy and Featherstone (35) used both brain homogenates and a brain hash which, they state, is histologically similar to a slice. Neither of these preparations was inhibited by N_2O using glucose or pyruvate as the substrate. The low degree of inhibition obtained by us with homogenates in the presence of glucose is comparable to the results reported by the above mentioned workers. However, it is questionable whether it is valid to compare the respiratory activity of a brain hash with that of a brain slice. The inhibition caused by N_2O seems to vary greatly with the mode of preparation of the tissue. It is possible that in the process of preparing a hash the normal enzyme sequence is disrupted as occurs

during homogenization. The N_2O-sensitive step, therefore, may possibly be by-passed in the resulting tissue preparation.

The question of the distribution of the gas also arises since the hash specimens must be of variable thickness. Unless the gas penetrates the tissue material uniformly, the degree of inhibition of respiration will be variable.

The Effect of Nitrous Oxide on Brain Respiration in the Presence of Methylene Blue

The mode of action of methylene blue acting as an electron carrier has already been discussed on page 38. It terminates the chain of reactions described on page 31 at a specific place. The use of this dye is very helpful for ascertaining where the inhibition occurs or whether the site of the inhibition is by-passed by methylene blue.

The concentration of methylene blue used in our experiments was 1×10^{-5} M. All the experiments discussed in the previous section were repeated with methylene blue. The results are presented on Table II.

(a) Succinoxidase System

In the succinoxidase system methylene blue accepts electrons from reduced cytochrome b, thus by-passing the entire cytochrome c-cytochrome oxidase system.

The respiration of brain slices in the presence of 0.01 M succinate was decreased by 56% in the presence of

N_2O. With the homogenates, however, the inhibition was only 12%.

Table II

The Effect of Nitrous Oxide on the Respiration of Rat Brain Slices and Homogenates in the Presence of Methylene Blue.
(Medium KRPG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	67	21
Succinate	56	12
Lactate	55	29
Pyruvate	83	38
Iso-citrate	60	16

For values see Appendices iii and iv

(b) DPN-linked Systems

In the DPN-linked systems methylene blue accepts electrons or hydrogen from the reduced flavoprotein.

(i) No Added Substrate

The inhibition by N_2O of the respiratory activity of

slices without added substrate was 67%, while with the homogenates it was only 21%.

(ii) Lactate

The inhibition produced by N_2O on brain slices respiring in the presence of lactate was 55%, while with the homogenates the inhibition was 29%.

(iii) Pyruvate

The respiration of brain slices in the presence of 0.01 M pyruvate was inhibited to the extent of 83% by the N_2O. The inhibition with homogenates was only 38%.

(c) TPN-linked Systems.

In the systems that require TPN as a coenzyme, methylene blue accepts electrons or hydrogen from TPN-cytochrome c reductase.

(i) Iso-Citrate

The iso-citrate system in the brain slices was inhibited by N_2O to an extent of 60%. In the homogenate, however, the inhibition was only 16%.

DISCUSSION

The studies with brain slices using methylene blue as a carrier show that the range of inhibition produced by N_2O

was between 55% and 83% depending on the substrate used. This range of inhibition is close to that obtained in the experiments (cf. page 77) in which no carrier was used, except in the case of pyruvate and of no added substrate. The reason for this is not clear.

In the case of the homogenates, the inhibition was much lower ranging between 12% and 38%. Here again the highest inhibition , 38%, was obtained in the presence of pyruvate.

In the succinoxidase system, methylene blue accepts electrons directly from cytochrome b, by-passing the cytochrome c cytochrome oxidase system. Since the inhibition observed both with and without methylene blue was the same, it may be inferred that the N_2O-sensitive step is either at the cytochrome b or at the succinic dehydrogenase stage.

In the DPN-linked systems, methylene blue accepts electrons from reduced flavoprotein. The sensitive component or components, therefore, must precede this stage.

In the TPN-linked system, methylene blue accepts electrons from TPN-cytochrome c reductase. The inhibition in this system is, therefore, either at this enzyme, at the TPN stage, or at the dehydrogenase stage.

Since in all these systems the inhibition of respiration occurs before the cytochrome c stages, it may be concluded that the entire cytochrome c-cytochrome oxidase system is not sensitive to N_2O.

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Brain

Since nitrous oxide inhibits the aerobic respiration of brain tissue, it was of interest to determine whether the anaerobic system also was sensitive to it.

In the experiments described below the ferricyanide technique of Quastel and Wheatley (82) was used. The experiments were carried out in a Krebs-Ringer-Bicarbonate-Glucose medium, which was previously gassed with CO_2. The gas phase was either 95% N_2-5% CO_2 or 95% N_2O-5% CO_2. The results of the experiments are presented in Table III.

(a) Succinic Dehydrogenase

When the succinoxidase system is studied under anaerobic conditions with the ferricyanide method, the CO_2 evolution is a measure of the dehydrogenase activity. The ferricyanide accepts electrons directly from the reduced succinic dehydrogenase. In this manner a single step of the electron transport pathway is measured.

The addition of 0.01 M succinate to the brain slices or homogenates metabolizing anaerobically, produces a great increase in the CO_2 evolution. The presence of N_2O in the gas phase does not alter the increased CO_2 evolution either in the slices or in the homogenates.

TABLE III

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Brain Slices and Homogenates (Ferricyanide Technique).

(Medium KRBG)

	Percentage Inhibition	
Substrate	Slices	Homogenate
No Added Substrate	20	15
Succinate	+4	+9
Lactate	19	15
Pyruvate	28	36
Iso-citrate	26	18

For values see Appendices v and vi

(b) DPN-linked Systems

In the dehydrogenase systems which require DPN as a co-enzyme, the ferricyanide accepts electrons from the reduced DPN. The amount of CO_2 evolved, therefore, indicates the rate of DPN reduction in the system.

(i) No Added Substrate

In the experiments without added substrate a 20% inhibition of respiration was observed with slices and 15% with homogenates in the presence of N_2O.

(ii) Lactate

The addition of 0.01 M lactate to metabolizing brain tissue did not produce an appreciable increase in CO_2 evolution. The inhibition produced by N_2O was 19% with slices and 15% with homogenates.

(iii) Pyruvate

The anaerobic metabolism of brain tissue was considerably increased in the presence of 0.01 M pyruvate. The metabolism was decreased by 28% when the slices were incubated in an N_2O-CO_2 atmosphere. In the case of the homogenate the inhibition in the presence of N_2O was 36%.

(c) TPN-linked System

In the systems in which TPN acts as the coenzyme and hydrogen carrier between the dehydrogenase and the flavoprotein, the addition of ferricyanide by-passes much of the chain by accepting electrons from the reduced TPN.

(i) Iso-citrate

The addition of 0.01 M iso-citrate to brain tissue did not greatly alter its metabolism. This system was inhibited to an extent of 26% in the slices and 18% in the homogenates.

DISCUSSION

The inhibition of the anaerobic metabolism of rat brain slices with different substrates varied between +4% and 28%,

that of homogenates between +9% and 36%. This range of inhibition is much lower than that previously observed with the aerobic respiration.

When the anaerobic metabolism of the succinoxidase system is studied with the ferricyanide method, the chain of reactions described on page 31 is interrupted at the dehydrogenase stage. The experiments described on page 86 show that the presence of N_2O does not inhibit the anaerobic system; in other words the succinic dehydrogenase is not sensitive to N_2O.

It has been observed in a previous section that the N_2O-sensitive component in the succinoxidase system must be either cytochrome b or succinic dehydrogenase. Since it has been shown that the succinic dehydrogenase is not sensitive to N_2O, it is reasonable to conclude that N_2O exerts its inhibitory effect by inactivating cytochrome b.

The anaerobic metabolism of the system requiring DPN as a coenzyme is inhibited slightly by N_2O. The inhibition ranging from 19% to 28% is much lower than that obtained with the aerobic system which ranges from 50% to 60%. It has already been shown that the inhibition by N_2O must occur at or preceding the flavoprotein stage. Since ferricyanide accepts electrons from reduced DPN, it means that more than 50% of the inhibition caused by N_2O occurs at the flavoprotein or diaphorase stage of the electron transport system.

The iso-citrate system, which requires TPN, shows the same type of inhibition as the above. The aerobic respiration of

brain slices in the presence of iso-citrate was reduced by 60%, while the anaerobic metabolism was decreased by 26%. It has already been shown that in this system, the inhibition must occur at or preceding the flavoprotein stage. Since the experiments with the aid of ferricyanide showed a much lower inhibition it follows that more than 50% of the total inhibition must occur at the TPN-cytochrome c reductase step.

The fact that there was a low inhibition of the anaerobic metabolism in both the DPN and the TPN linked systems, indicates that there are two or more N_2O-sensitive components in the electron transport system. Either the dehydrogenase step is also inhibited by N_2O, or the coenzyme is the sensitive factor. It is possible also that both these components are sensitive to some degree.

It is evident that the inhibition of respiration in the homogenate under anaerobic conditions is comparable with that of the slices. It has been shown that under aerobic conditions, the inhibition with homogenates was always much lower than with slices. Considering that the inhibition occurs at more than one stage the above observations can now be explained. It is possible that in the homogenates under aerobic conditions one of the N_2O-sensitive steps is by-passed thus reducing the inhibition but not eliminating it altogether since some of the other steps also are sensitive. By comparing the degree of inhibition in homogenates under aerobic with that under anaerobic conditions, it is evident that a close agreement exists between the two. It seems, therefore, that the same enzymes in the homogenate are inhibited under aerobic as under anaerobic conditions.

The Effect of Nitrous Oxide on the Respiration of Brain Tissue in the Presence of Added DPN

The following experiments were carried out to ascertain the effect of adding extra DPN to brain homogenate under aerobic and under anaerobic conditions since the possibility arose that DPN could be sensitive to N_2O.

The Aerobic Respiration of Brain Homogenate

In order to determine whether the reduction of DPN is sensitive to N_2O extra DPN was added to the respiring tissue. If DPN should be the sensitive component, the addition of DPN should diminish or completely abolish the inhibition. In these experiments the brain homogenate respired in the presence of pyruvate, and 4.3×10^{-4} M DPN. As the results in Table IV indicate, no decrease in the inhibition occurred.

TABLE IV

The Effect of Nitrous Oxide on the Respiration of Brain Homogenate in the Presence of Added DPN
(Medium Without glucose)

System	Percentage Inhibition
Control	11
" + Pyruvate	31
" + " + DPN	38

For values see Appendix vii

Anaerobic Metabolism of Brain Homogenates

In these experiments, the effect of extra DPN on the anaerobic metabolism of rat brain homogenates was studied.

The addition of 6.5×10^{-4} M DPN to brain homogenates metabolizing lactate or pyruvate or without added substrate as indicated in Table V does not produce a significant decrease in the inhibition.

TABLE V

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Brain Homogenate in the Presence of Added DPN (Medium KRBG)

System	Percentage Inhibition
Control	25
" + Lactate	33
" + Pyruvate	31
" + DPN	29
" + Lactate + DPN	25
" + Pyruvate + DPN	26

For Values see Appendix viii

DISCUSSION

It is apparent that the addition of extra DPN does not abolish or greatly decrease the inhibition produced by N_2O.

The reduction of DPN, therefore, is not the N_2O-sensitive reaction in the respiratory chain. The only alternative, therefore, is that the N_2O-sensitive component in the DPN-linked system must be the dehydrogenase itself. Thus, it may be concluded that lactic dehydrogenase is slightly sensitive to N_2O.

STUDIES ON LIVER AND KIDNEY

Jowett and Quastel (43) observed that certain drugs which decreased the metabolism of brain also inhibit the respiration of other tissues.

In view of the observed inhibition of the metabolism of brain tissue by N_2O it was of interest to see whether the same enzyme systems of liver and kidney also are sensitive to the gas.

The Effect of Nitrous Oxide on the Respiration of Liver and Kidney

The techniques and procedures in the experiments were the same as those used in the study of brain tissue. Both sliced and homogenized preparations were studied. The results are presented in Tables VI and VII.

(a) Succinoxidase System

The addition of 0.01 M succinate to liver and kidney tissue preparations produced a marked stimulation of the

respiratory activity.

The respiration of liver slices and homogenates in the presence of succinate was inhibited by N_2O to an extent of 40%.

With kidney tissue the inhibition produced by N_2O was in the same range as the above and the same for both slices and homogenates.

TABLE VI

The Effect of Nitrous Oxide on the Respiration of Rat Liver Slices and Homogenates (Medium KRPG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	68	8
Succinate	40	40
Lactate	57	9
Pyruvate	72	0
Iso-citrate	60	0

For Values see Appendices ix and xi

(b) DPN-linked Systems

The DPN-linked systems as studied with brain tissue were reinvestigated with liver and kidney tissue.

(i) No Added Substrate

The oxygen uptake by liver slices without added substrate was inhibited to the extent of 68% in the presence of N_2O. The homogenates, however, were practically unaffected.

With kidney, the respiratory activity of the slices also was inhibited 66%, while the inhibition with homogenates again was very low, 7%.

(ii) Lactate

The addition of 0.01 M lactate to liver and kidney tissue caused an increase in the O_2 consumption.

N_2O decreased the respiration of liver slices, in the presence of lactate by 57%, while that of homogenates was only decreased by 9%.

With kidney tissue the inhibition was 65% with slices, but only 8% with homogenates.

(iii) Pyruvate

The respiration of liver tissue was stimulated on the addition of 0.01 M pyruvate. Nitrous oxide inhibited the oxygen consumption of slices by 72%. In homogenates, no inhibition at all was produced.

The respiration of kidney tissue was also slightly stimulated by the addition of pyruvate. In the presence of N_2O the inhibition with slices was 54%, and with homogen-

ates only 12%.

TABLE VII

The Effect of Nitrous Oxide on the Respiration of Rat Kidney Slices and Homogenates (Medium KRPG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	66	7
Succinate	41	45
Lactate	65	8
Pyruvate	54	12
Iso-citrate	71	+3

For values see Appendices x and xii

(c) TPN-linked Systems

The TPN-linked system investigated was the same as that previously studied with brain.

(i) Iso-citrate

The addition of 0.01 M iso-citrate to respiring liver tissue did not greatly alter the oxygen consumption. The respiration of liver slices was inhibited 60% by N_2O, while

with homogenates there was no inhibition.

The respiration of kidney slices and homogenates was not greatly increased in the presence of iso-citrate. Nitrous oxide inhibited this system in slices to an extent of 71%, but caused no inhibition with homogenates.

DISCUSSION

The results of these experiments indicate that nitrous oxide inhibits the respiratory activity of rat liver and kidney slices. The inhibition varied slightly with the nature of the added substrate.

With liver slices the lowest inhibition was obtained with succinate (40%), while with the other substrates, the degree of inhibition ranged between 57% and 72%.

With liver homogenates, N_2O caused no inhibition of the respiration in the presence of any of the substrates except succinate. The oxygen uptake of both slices and homogenates in the presence of succinate was inhibited 40%. It may be concluded, therefore, that the factor or factors sensitive to N_2O in the succinoxidase system cannot be by-passed in the homogenate as is the case with other substrates.

The inhibition of the respiratory activity in the presence of the other substrates was negligible. The difference between the inhibition in slices and that in homogenates has already been mentioned with reference to brain tissue. The same explanation as given previously probably holds here, namely that

the factor or factors sensitive to N_2O are by-passed or destroyed in the homogenate.

The degree of inhibition of the oxygen uptake by N_2O in kidney slices was about the same as with liver slices. The lowest inhibition [41%] was obtained when succinate was the substrate. The inhibition with the other substrates ranged between 54% and 71%.

The inhibition of the respiratory activity of kidney homogenates by N_2O was very slight except in the presence of added succinate. The oxygen uptake in the presence of succinate and N_2O was decreased by 45%. This is comparable to the degree of inhibition in slices under similar conditions. It is apparent, therefore, that in both kidney and liver homogenate, the N_2O-sensitive step cannot be by-passed.

The inhibition of the oxygen uptake in kidney tissue in the presence of other substrates was found to be negligible. The sensitive component of the respiratory system, therefore, may be by-passed in the kidney homogenate, as was previously observed with liver and brain.

The Effect of Nitrous Oxide on the Respiration of Liver and Kidney in the Presence of Methylene Blue

The purpose of using methylene blue was to locate the site of the inhibition produced by nitrous oxide. The results of the experiments are presented in Table VIII and IX.

(a) Succinoxidase System

The respiration of liver slices and homogenates with succinate as the substrate in the presence of methylene blue was inhibited by nitrous oxide. The inhibition with slices was 52% and that with homogenates 35%.

Nitrous oxide was also observed to inhibit the respiratory activity of kidney cortex slices [48%] and that of kidney cortex homogenates [45%].

TABLE VIII

The Effect of Nitrous Oxide on the Respiration of Rat Liver in the Presence of Methylene Blue (Medium KRPG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	61	0
Succinate	52	35
Lactate	52	+8
Pyruvate	58	2
Iso-citrate	65	12

For values see Appendices xiii and xv

(b) DPN-linked Systems

(i) No Added Substrate

The respiratory activity of liver slices in the presence of methylene blue without added substrate was inhibited by N_2O to an extent of 61%. The oxygen uptake by the homogenate remained unchanged under the same conditions.

The inhibition of the respiratory activity of kidney cortex slices was 62%, while that of kidney homogenates was only 10%.

(ii) Lactate

The degree of inhibition of the respiratory activity of liver slices caused by N_2O, with lactate as the substrate and in the presence of methylene blue was 52%. With liver homogenates, on the other hand, there was a slight increase in the oxygen uptake.

Nitrous oxide inhibited the respiration of kidney slices 61%. With the homogenates, however, the respiration was unaffected.

(iii) Pyruvate

Liver slices respiring in the presence of pyruvate and methylene blue were inhibited by N_2O to an extent of 58%. There was no considerable inhibition of the respiration in the homogenates.

With kidney slices the inhibition was 71%, while with homogenates no inhibition was observed.

TABLE IX

The Effect of Nitrous Oxide on the Respiration of Rat Kidney in the Presence of Methylene Blue (Medium KRPG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	62	10
Succinate	48	45
Lactate	61	+5
Pyruvate	71	0
Iso-citrate	72	0

For values see appendices xiv and xvi

(c) TPN-linked Systems

(i) Iso-citrate

Nitrous oxide inhibited the oxygen consumption of liver slices in the presence of iso-citrate by 65%. With homogenates the inhibition was only 12%.

The respiration of kidney slices was inhibited to an extent

of 72%, while kidney homogenates showed no inhibition.

DISCUSSION

It is evident from the foregoing data that the inhibition produced by N_2O is the same in the presence and in the absence of methylene blue, except in the case of pyruvate where it is slightly higher with methylene blue. This behaviour is similar to that observed with brain tissue.

The activity of the succinoxidase system of both liver and kidney was decreased to about one half in the presence of N_2O and the dye. This was observed with both slices and homogenates. The inhibition, therefore, must precede the oxidation of reduced cytochrome b in the respiratory system. The sensitive step cannot be by-passed in the homogenate.

In the DPN-linked systems, nitrous oxide inhibits, as in the case of brain tissue, some enzyme or enzymes preceding the cytochrome c - cytochrome oxidase system in the respiratory chain. The N_2O sensitive components in liver and kidney tissue homogenates, however, seem to be completely by-passed.

The TPN-linked system exhibits the same degree of inhibition, which occurs only in slices and precedes the oxidation of reduced TPN-cytochrome c reductase.

ANAEROBIC STUDIES ON LIVER AND KIDNEY

In view of the profound effect of N_2O on the respiration of

rat liver and kidney tissue, it was of interest to study the influence of the gas on the anaerobic metabolism of these tissues.

All the experiments that had previously been carried out under aerobic conditions were repeated under anaerobic conditions using the ferricyanide method of Quastel and Wheatley (82). The results are presented in Tables X and XI.

(a) Succinic Dehydrogenase

The succinic dehydrogenase of liver is not sensitive to N_2O. With slices or with homogenates no inhibition was produced by N_2O.

Studies with kidney tissue showed that the dehydrogenase of that organ also is insensitive to N_2O. The metabolism of kidney slices was inhibited only 6%, which is negligible, and the homogenates were not inhibited at all.

TABLE X

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Liver (Ferricyanide Technique)

(Medium KRBG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	9	12
Succinate	+3	+3
Lactate	8	13

TABLE X (continued)

	Percentage Inhibition	
Substrate	Slices	Homogenates
Pyruvate	6	9
Iso-citrate	14	16

For values see Appendices xvii and xviii

(b) DPN-linked Systems

(i) No Added Substrate

The liver slices and the homogenates metabolizing under anaerobic conditions without added substrate were inhibited by N_2O, 9% and 12% respectively.

The inhibition was only slightly greater with kidney slices, 17%, and with kidney homogenates, 19%.

(ii) Lactate

The inhibition produced by N_2O on the metabolism of lactate by liver slices and homogenates was very small, namely, 8% and 13% respectively.

Kidney slices metabolizing lactate in the presence of N_2O were inhibited 13%. The inhibition with homogenates

was 24%.

(iii) Pyruvate

Nitrous oxide did not inhibit the metabolism of pyruvate by liver slices and homogenates very much. The inhibition with slices was 6% and with homogenates 9%.

In the case of kidney tissue, however, the inhibition produced by N_2O was greater. The activity of the kidney slices was inhibited by 34%, and that of the homogenates by 27%.

TABLE XI

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Kidney (Ferricyanide Technique) (Medium KRBG)

	Percentage Inhibition	
Substrate	Slices	Homogenates
No Added Substrate	17	19
Succinate	6	0
Lactate	13	24
Pyruvate	34	27
Iso-citrate	24	15

For values see Appendices xix and xx

(c) TPN-linked Systems.

(i) Iso-citrate

The iso-citrate system of liver tissues was inhibited by 14% in the case of slices, and 16% in the case of homogenates in the presence of N_2O.

In the kidney, the inhibition with slices was 24% and with homogenates was 15%.

DISCUSSION

The results of the experiments just described show that succinic dehydrogenase of both liver and kidney is not sensitive to N_2O. In view of the results obtained in the aerobic studies of the succinoxidase system of liver and kidney, it may be concluded that the N_2O-sensitive factor is cytochrome b. Studies of the brain succinoxidase system on page 86 have shown that cytochrome b is the sensitive component of that system also.

The DPN-linked systems of liver tissue were only slightly inhibited under anaerobic conditions. This inhibition ranged from 6% to 13% and thus was within the experimental error. It may be said, therefore, that almost the entire inhibition under aerobic conditions occured after the DPN stage and before the BAL-labile factor in the metabolic sequence. This means that the N_2O sensitive component was the diaphorase.

The DPN-linked systems of kidney show only a slight

inhibition under anaerobic conditions. Hence, it seems that in the kidney there is an additional element which is sensitive to N_2O. Most of the inhibition in this tissue also occurs at the flavoprotein step. The rest of the inhibition must either involve the DPN or the dehydrogenase.

The TPN-linked iso-citrate system of both liver and kidney showed a small inhibition ranging from 14% to 24%. This inhibition must involve either the TPN or the dehydrogenase. Most of the inhibition occurs at the TPN-cytochrome c reductase step.

In these anaerobic studies, the inhibition obtained with slices was similar to that obtained with homogenates. The system that was inhibited in slices, therefore, was not by-passed in the homogenate.

The Recovery from Nitrous Oxide Inhibition

The recovery of metabolic activity after inhibition by nitrous oxide of the oxidation of succinate by liver and kidney homogenates was studied. The respiration of the homogenate was measured for 30 minutes in the presence of 80% N_2O:20% O_2. Air was then passed through the manometers for ten minutes and the respiration was again measured for 30 minutes. The results are presented in Table XII.

DISCUSSION

The recovery from the effects of nitrous oxide was rapid

TABLE XII

The Recovery from Nitrous Oxide Inhibition in Liver and Kidney Homogenates

System	µl. O_2 taken up		
	First 30 minutes		Second 30 minutes Regassed with Air
	Air	80%N_2O:20%O_2	
LIVER			
Controls			
Succinate	330	-	-
" + MB*	376	-	-
Experimental			
Succinate	-	157	293
" + MB	-	212	292
KIDNEY			
Controls			
Succinate	375	-	-
" + MB	425	-	-
Experimental			
Succinate	-	167	374
" + MB	-	216	354

Experimental conditions as in Appendices ix to xvi

* MB = Methylene blue

and practically complete *in vitro*. These findings were identical with those from experiments performed on animals *in vivo*. The results of the two sets of experiments indicate quite clearly that air reverses the effects of N_2O just as efficiently in the intact animal as in the isolated tissue.

SUMMARY

The experiments described in this section have shown that certain components of the respiratory chain are sensitive to nitrous oxide. The results may be summarized as follows:

1. The entire cytochrome *c* - cytochrome oxidase system of brain, liver and kidney is not sensitive to nitrous oxide.
2. The N_2O-sensitive component of the succinoxidase system in all the three tissues is cytochrome *b*.
3. In both the DPN and the TPN-linked systems, the flavoprotein is sensitive to nitrous oxide. In the DPN-linked system it is the diaphorase while in the TPN-linked system it is the TPN-cytochrome *c* reductase.
4. The reduction of DPN is not inhibited by nitrous oxide.
5. The lactic dehydrogenase of both brain and kidney and the iso-citric dehydrogenase are only slightly sensitive to N_2O.
6. Lactic and iso-citric dehydrogenases of liver are not

N_2O-sensitive.

7. Succinic dehydrogenase of brain, liver and kidney is not N_2O-sensitive.
8. Under aerobic conditions, the degree of inhibition is lower with homogenates than with slices.
9. The inhibition produced by N_2O on the respiration of liver and kidney is rapidly and practically completely reversed by the removal of the anesthetic gas.

GLYCOLYSIS

It was found by Meyerhof and his associates (84, 86) that brain extracts are capable of a high rate of glycolysis, and are more efficient than whole homogenates. The extracts have to be reinforced with ATP, but since the amount of ATP-ase in them is very low, very little of the ATP is lost by hydrolysis. The rate of glycolysis in the extracts is determined by the amount of ATP added. Meyerhof et al. (85) found that the amount of lactic acid formed as the product of glycolysis, corresponded closely to the volume of CO_2 evolved during the process. He pointed out that some of the CO_2 was evolved as the result of the formation of phosphoglyceric acid.

The Effect of Nitrous Oxide on Anaerobic Glycolysis

The experiments described in this section show the effect of nitrous oxide on the glycolytic system of rat brain extracts.

The results in Table XIII show that the anaerobic glycol-

ysis was inhibited significantly by nitrous oxide. When the concentration of ATP was increased, the rate of glycolysis increased greatly. The degree of inhibition produced by nitrous oxide, however, remained unchanged. This implies that the nitrous oxide does not interfere with the utilization of ATP in the different reactions of the glycolytic system.

TABLE XIII

The Effect of Nitrous Oxide on the Anaerobic Glycolysis of Rat Brain Extracts

System	Percentage Inhibition
Control + glucose	70
" + " + 3×10^{-4} M ATP	71
" + glucose + 6×10^{-4} M ATP	78
" + glucose + 10×10^{-4} M ATP	69

For values see Appendix xxi

The percentage inhibition in all the experiments on anaerobic glycolysis was calculated on the basis of the QO_2 evolved during a 30 minute period, since it was found that after this time the CO_2 evolution in the system gassed with

$N_2O:CO_2$ had ceased.

When it was found that nitrous oxide inhibits anaerobic glycolysis, it was of interest to see whether the inhibition could be diminished or altogether abolished by the addition of various intermediates of the glycolytic system. Accordingly, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate respectively, were used as substrates. The results are presented on Table XIV.

TABLE XIV

The Effect of Nitrous Oxide on Anaerobic Glycolysis Using Various Substrates

Substrate	Percentage Inhibition
Glucose	72
Glucose-6-phosphate	74
Fructose-6-phosphate	75
Fructose-1,6-diphosphate	73

For values see Appendix xxii

The systems employed for the above experiments all contained 10^{-3} M ATP, which concentration was found to favor the maximum rate of glycolysis. The results indicate that

the inhibition produced by N_2O when the above mentioned intermediates of the glycolytic systems were used was the same as with glucose.

In an effort to locate the site of inhibition in the rest of the glycolytic sequence, it was of interest to carry out some experiments using 3-carbon imtermediates. A few of the available ones were tried, but no conclusions could be drawn from these experiments. The results have, therefore, been omitted.

Recovery from the Effects of Nitrous Oxide

The reversal of the inhibition produced by N_2O was studied in another set of experiments.

The glycolytic rate in the presence of N_2O:CO_2 was first measured during a period of 30 minutes. Following this, the vessels were regassed with 95% N_2:5% CO_2 for 10 minutes, and the glycolytic activity was again measured during a 30 minute period. It was found that the system remained inactive after regassing. However, if ATP was added to the medium and the manometers were regassed, the system that had been previously inhibited, now rapidly resumed its activity. The results are presented in Table XV and Figure 2.

TABLE XV

The Recovery of the Glycolytic Activity of Rat Brain Extracts (RBE) after Nitrous Oxide Inhibition

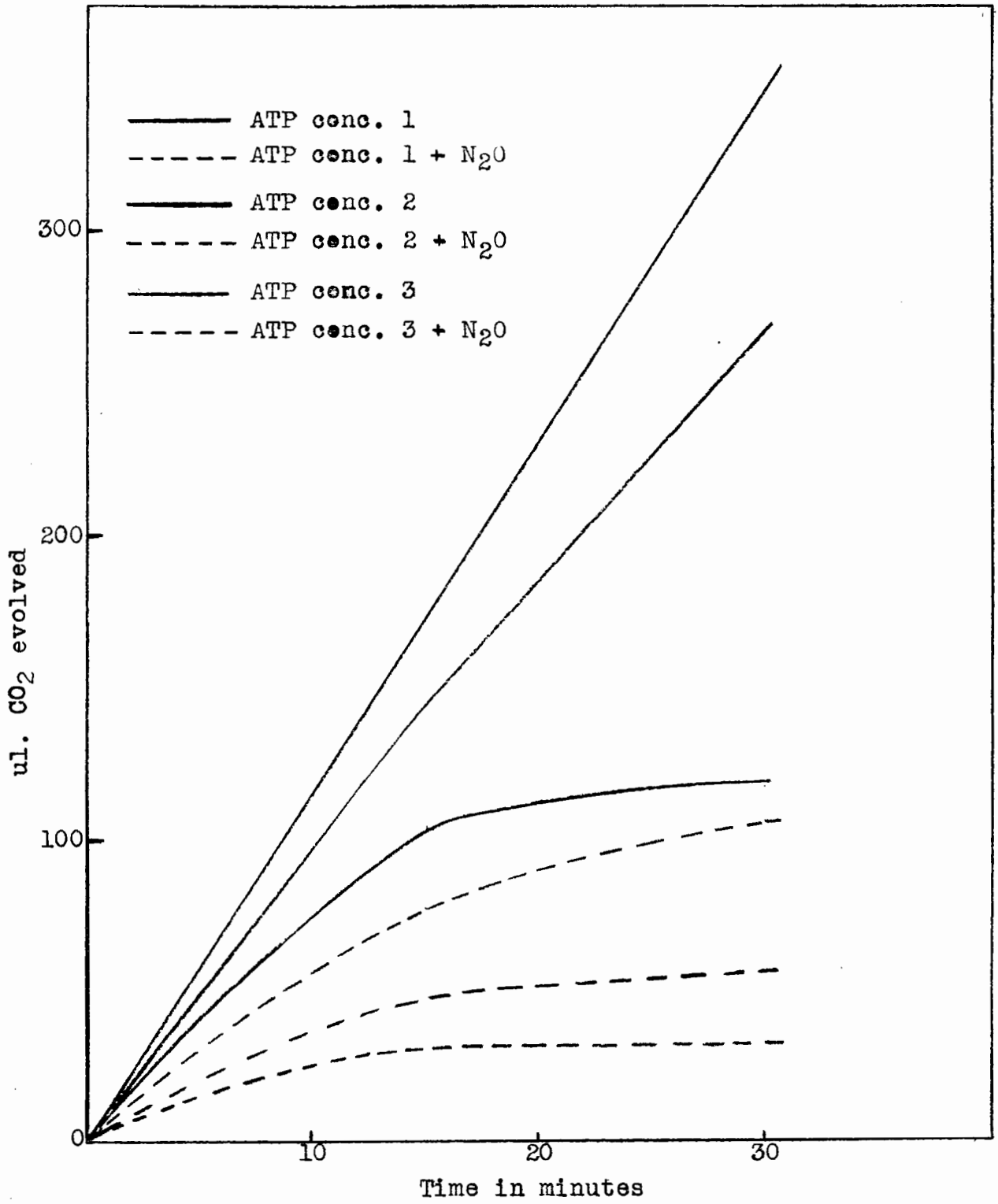
System	μl. CO_2 evolved		
	First 30 minutes		Second 30 Minutes
	N_2:CO_2	N_2O:CO_2	N_2:CO_2
Controls:			
RBE + Glucose + ATP_1	350	-	-
RBE + Glucose + ATP_2	455	-	-
Experimental			
RBE + Glucose + ATP_1	-	84	358
RBE + Glucose + ATP_2	-	107	391

All values are corrected for blanks

ATP_1 = 6 x 10^{-4} M

ATP_2 = 10 x 10^{-4} M

Experimental conditions as in Appendices xxi and xxii



The Effect of Nitrous Oxide on the Anaerobic Glycolysis of Rat Brain Extracts.

Fig. 2

DISCUSSION

The experiments described in this section show that nitrous oxide produces a significant inhibition of the glycolytic system of rat brain extracts. This would imply that in the course of $N_2O:O_2$ anesthesia, the glycolytic activity of brain tissue is greatly decreased, thus decreasing the formation of energy-rich compounds.

The phosphorylation of glucose by hexokinase, requires ATP. This reaction is not sensitive to N_2O, since the addition of glucose-6-phosphate as the substrate did not diminish the degree of inhibition.

The next step studied in the glycolytic sequence was the isomerization of glucose-6-phosphate to fructose-6-phosphate. This reaction is catalyzed by oxoisomerase, also called phosphohexoisomerase. The enzyme does not require a cofactor. Nitrous oxide had no effect on this reaction, since, when either glucose-6-phosphate or fructose-6-phosphate was used, the same degree of inhibition was obtained.

The phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate by phosphohexokinase, requires ATP as the phosphate donor. This reaction is analogous to the phosphorylation of glucose by hexokinase. This step also was not sensitive to N_2O.

Since all the steps in the glycolytic sequence, from glucose to hexose diphosphate, were inhibited to the same ex-

tent by N_2O, the inhibition must occur at some reaction or reactions involving the 3-carbon intermediates.

The phosphorylation of ADP to yield ATP takes place at two points in the glycolytic sequence, namely, the formation of 3-phosphoglyceric acid from 1,3-diphosphoglyceric acid, and the formation of pyruvic acid from 3-phosphopyruvic acid. Levy and Featherstone (35) have shown in other systems that the formation of ATP is not sensitive to N_2O. It may, therefore, be concluded that the inhibition of glycolysis is not a result of the accumulation of ADP. This conclusion is confirmed by the fact that the addition of increasing amounts of ATP did not alter the degree of inhibition. It is not the lack of ATP, therefore, that causes the decreased glycolytic activity.

It was found from experiments described in the previous section, that lactic dehydrogenase was slightly sensitive to N_2O. However, this sensitivity accounts for less than one third of the total inhibition of glycolysis. Previous experiments also showed that DPN was not sensitive to N_2O.

Since the dehydrogenases studied so far have not shown very great sensitivity to N_2O, it seems reasonable to conclude that triosephosphate dehydrogenase is not inhibited to any significant degree.

Nitrous oxide must exert its effect on one or more of the remaining enzymes of the glycolytic chain, but, as has

already been pointed out, no conclusion can be drawn as to the site of inhibition.

The results presented in Table XV indicate that the inhibition produced by N_2O on the glycolysis of brain extracts, can be very readily reversed by the removal of N_2O from the gas phase and by the addition of extra ATP. Meyerhof et al. (85) found that there is enough ATP-ase in the brain extract to hydrolyze all of the added ATP if the system is not provided with a substrate. They showed that the controls incubated for the same period as the vessels containing the complete system, including the substrate, displayed only feeble glycolytic activity, and that all of the added ATP was hydrolyzed. The results obtained in our experiments can be explained in the same way. Since in the system inhibited by N_2O the glycolytic activity was low, much of the ATP must have been hydrolyzed by the action of the ATP-ase. At the end of the 30 minute period, therefore, the amount of ATP remaining in the system was insufficient to support glycolysis after the inhibitor was removed. When additional ATP was supplied, however, the system was capable of very high glycolytic activity.

In the studies on respiration [cf. page107] it was found that the inhibition produced by N_2O could be very easily reversed. The results obtained from the experiments on anaerobic glycolysis show that the inhibitory action of N_2O was just as easily counteracted by replacing the N_2O by N_2.

SUMMARY

The results of the experiments described in this section may be summarized as follows:

1. Nitrous oxide significantly inhibits the anaerobic glycolysis of rat brain extracts.
2. Increasing the amount of ATP does not alter the inhibition.
3. Hexokinase and phosphohexokinase are not sensitive to nitrous oxide.
4. Oxoisomerase or phosphohexoisomerase is not sensitive to nitrous oxide.
5. Nitrous oxide must exert its inhibition on one or more steps following the formation of fructose-1,6-diphosphate in the glycolytic sequence.

ACETYLATIONS

The use of sulfanilamide in measuring the activity of acetylating systems was introduced by Klein and Harris (182) in 1938. The procedure has now become a standard method. In 1946, Lipmann and Kaplan (183) prepared a pigeon liver acetone powder which contained the enzyme necessary for the acetylation of sulfanilamide. They found that this system gave maximum activity when it was fortified with acetate, ATP, CoA and cysteine.

The acetylsulfanilamide formed as a result of the acetyl-

ation of sulfanilamide is very stable. Sulfanilamide can be estimated by the very sensitive chemical method already described on page 67, and the acetylsulfanilamide can be determined by difference.

Johnson (184) has found that acetylsulfanilamide is not broken down by brain homogenates, and refers to Lipmann's work showing that pigeon liver extracts do not hydrolyse the ester. Hence, the extent of acetylation can be measured quite accurately.

It was found by Johnson (184) that pigeon liver acetone powder will acetylate sulfanilamide when acetate and ATP are present, without the addition of CoA. The rate of synthesis is constant and does not decline with increased concentration of acetylsulfanilamide. However, the addition of acetate alone results in a very low rate of acetylation.

The Effect of Nitrous Oxide on the Acetylation of Sulfanilamide

The acetylating system used was a pigeon liver acetone powder extract (PL) prepared by the method described on page 65. The incubation medium used contained the following components:

0.03 M KCl	0.03 M $MgCl_2$
0.05 M NaF	0.005 M Nicotinamide

0.01 M phosphate buffer pH 7.5

105 μg. sulfanilamide were added to each vessel

0.7 ml of PL (90 mg. powder) was incubated in the above

medium at 37.5°C. for 60 minutes. The acetylated sulfanilamide was then measured.

TABLE XVI

The Effect of Nitrous Oxide on the Acetylation of Sulfanilamide by Acetate

	μg. sulfanilamide acetylated	
System	Air	80%N_2O:20%O_2
PL	2	0
PL + 0.02 M Acetate	12	14
PL + 0.02 M Acetate + 0.003 M ATP	79	77

The results in Table XVI indicate that pigeon liver alone was not capable of acetylation. The addition of acetate produced a slight amount of acetylation. However, the addition of ATP resulted in a highly active acetylating system. These results are in agreement with those of Johnson.

The results in Table XVI also indicate that the acetylating mechanism was not affected by the presence of nitrous oxide in the vessels. The acetylating process is essentially an anaerobic one, since there was no uptake of oxygen in either system, whether the gaseous phase was air or 80%N_2O: 20%O_2.

The Effect of Nitrous Oxide on the Aerobic Acetylation of Sulfanilamide

It was found by Johnson (184) that by combining the sulfanilamide acetylating system of pigeon liver with that of rat brain homogenates both acetylation and oxidation could be studied together. By adjusting the ratio of the quantity of brain homogenate to that of pigeon liver extract, and by adding fluoride to the medium, the rate of acetylation could be increased to a maximum.

The rat brain homogenate is incapable of acetylating aromatic amines, and serves as the respiring system which supplies the necessary energy. The pigeon liver extract does not respire, but contributes only the acetylating system.

The results in Table XVII indicate that the respiration of rat brain homogenate was inhibited by N_2O to a small extent. The acetylating system of pigeon liver also was inhibited both in the absence and presence of acetate. The addition of ATP decreased the inhibition. The entire range of inhibitions was very low.

TABLE XVII

The Effect of Nitrous Oxide on the Aerobic Acetylation

	Percentage Inhibition	
System	Respiration	Acetylation
PL + RBH	18	16
PL + RBH + Acetate	19	16
PL + RBH + Acetate + ATP	8	6

For values see Appendix xxiii

The symbols used in Table XVII represent

Pigeon Liver Extract = PL

Rat Brain Homogenate = RBH

In the next set of experiments, pyruvate was used as the acetyl donor. Johnson (184) has shown that pyruvate, under aerobic conditions, gives rise to a high rate of acetylation of the sulfanilamide.

TABLE XVIII

The Effect of Nitrous Oxide on the Respiration and Acetylation of Rat Brain Homogenate (RBH) and Pigeon Liver Extract (PL) in the Presence of Pyruvate

	Percentage Inhibition	
System	Respiration	Acetylation
PL + RBH	15	31
PL + RBH + Pyruvate	34	54
PL + RBH + Pyruvate + DPN	39	51
PL + RBH + Pyruvate + ATP	43	37
PL + RBH + Pyruvate + DPN + ATP	43	42

For values see Appendix xxiv

The results in Table XVIII show that the oxidation of pyruvate by rat brain homogenate was inhibited by nitrous oxide. The addition of either DPN or ATP did not decrease the inhibition. The rate of acetylation of sulfanilamide also

was inhibited by nitrous oxide. The addition of DPN did not change the inhibition, but the addition of ATP slightly diminished it.

DISCUSSION

The sulfanilamide acetylating system present in pigeon liver is capable of maximum activity only when an acetyl donor and ATP are added. This system is strictly an anaerobic one, since there is no appreciable oxygen uptake even in the presence of oxygen. The observation that nitrous oxide does not inhibit this system indicates that the sulfanilamide acetylating enzyme is not sensitive to that gas.

When the acetylating system was coupled with the oxidative system of rat brain homogenate, the results were different. The oxygen uptake of the entire system decreased and the rate of acetylation also was inhibited. The addition of pyruvate to the system increased the rate of oxidation and acetylation slightly. However, the inhibition of respiration and acetylation by the nitrous oxide was greatly increased.

Studies on the hydrogen transport system [cf.page 75] have shown that the metabolism of pyruvate is inhibited by nitrous oxide. One of the sensitive components of this system was shown to be the diaphorase. The inhibition of the pyruvate metabolism remained the same when either DPN or ATP, or both, were added to the system.

The dismutation of pyruvate supplies the precursor necessary for the acetylation of sulfanilamide. DPN is a necessary

cofactor in the dismutation process. However, the addition of DPN to the system inhibited by nitrous oxide, did not in any way alter the inhibition.

It has been shown by Lipmann and Kaplan (183) that ATP is essential for the acetylation of sulfanilamide. Since a certain amount of sulfanilamide is acetylated in the presence of pigeon liver and rat brain homogenate, the homogenate must retain some ATP. On the addition of ATP, the rate of acetylation was greatly increased. The inhibition of acetylation by nitrous oxide fell from 54% to 40% when ATP was added. This difference is not very significant, nevertheless, it seems to indicate that the addition of ATP partially overcomes the inhibition. Johnson (184) has suggested that the inhibition produced by certain narcotics on the acetylating system of pigeon liver is the result of the inhibition of the oxidative synthesis of ATP, since it can be almost completely reversed by the addition of ATP. Levy and Featherstone (35) claim that N_2O does not inhibit oxidative phosphorylation of guinea pig brain homogenates. In view of these facts it seems that only a small part of the inhibition caused by N_2O on the acetylating system of pigeon liver is due to the lack of ATP and that the greater part of it must be the result of other N_2O-sensitive components. The results described in the section on glycolysis [cf.p.110] also indicate that the synthesis of ATP is not sensitive to N_2O.

The acetylation of sulfanilamide is dependent upon the dismutation of pyruvate. This system is inhibited by nitrous oxide, thus indicating that the amount of acetyl-CoA formed from the pyruvate is quite small. Since this acetyl-CoA is the factor necessary for the acetylation of sulfanilamide, the decrease in its formation will consequently decrease the amount of sulfanilamide acetylated. Hence, the inhibition produced by N_2O on the acetylating system is not attributable to the effect of that gas on any component of that system, but must be the result of the inhibition exerted on the reactions that supply its precursors.

This explanation is supported by the results [cf.p121] obtained when the rate of anaerobic acetylation in the presence of pigeon liver and acetate was measured. That system was not inhibited in the least by N_2O.

The aerobic acetylation in the presence of acetate was inhibited very slightly by N_2O. This feeble degree of inhibition is not significant. Since acetate is not metabolized but is used up directly in the acetylation of the sulfanilamide, the N_2O cannot exert its inhibitory effect on any of the preceding reactions. The small decrease observed in both acetylation and respiration was probably the result of an inhibition exerted on the endogenous substrates of rat brain homogenate.

SUMMARY.

The results obtained in these studies may be summarised as follows:

1. The anaerobic system responsible for the acetylation of sulfanilamide in pigeon liver extracts, is not sensitive to nitrous oxide.
2. The aerobic acetylation of sulfanilamide with acetate as the acetyl donor is slightly sensitive to nitrous oxide.
3. The aerobic acetylation of sulfanilamide resulting from the dismutation of pyruvate is N_2O-sensitive.
4. The addition of DPN to the sulfanilamide-acetylating and pyruvate dismutating systems does not alter the inhibition produced by nitrous oxide.
5. The addition of ATP to the above systems decreases the inhibition only slightly.
6. The addition of both DPN and ATP to the above systems gives the same result as the addition of ATP alone.

OTHER ENZYME SYSTEMS

A study of other enzyme systems was also carried out to determine whether any of them are sensitive to nitrous oxide.

The Effect of Nitrous Oxide on Choline Oxidase

The choline oxidase of rat liver was prepared according to the method of Bernheim and Bernheim (128) as follows:

> Freshly dissected rat liver was chopped with scissors and ground in a mortar with sand in 0.05 M phosphate buffer (pH 7.8). The macerated tissue was then pressed through sterile muslin and the volume of the expressed solution made up to 20 ml. with phosphate buffer. This mixture was dialysed against distilled water in the refrigerator for 24 hours. Precaution was taken throughout the treatment to limit the chance of bacterial contamination.
>
> The contents of the dialysing sac were then centrifuged and the precipitate suspended in a small amount of water and again centrifuged. The precipitate was finally suspended in twice its volume of phosphate or bicarbonate buffer according to the requirement of the experiment.

The choline oxidase and choline dehydrogenase activity of these preparations was measured by the method of Mann, Woodward and Quastel (129).

The results obtained when the activity of the above systems was measured in the presence of N_2O are presented in Tables XIX and XX. It is quite clear that neither the dehydrogenase nor the oxidase system was inhibited by N_2O.

TABLE XIX

The Effect of Nitrous Oxide on the Choline Dehydrogenase of Rat Liver

	μl. of CO_2 evolved/hour	
System	95% N_2:5% CO_2	95% N_2O:5% CO_2
Control (entire system without substrate)	14	21
Control + choline conc. 1	170*	185*
Control + choline conc. 2	252*	290*

* Corrected for blanks.

Flask Contents:

Main Compartment: 0.5 ml of enzyme**
1.3-1.8 ml of 0.025 M bicarbonate depending on additions from side bulb

Side Bulb: 0.1 ml choline chloride (in buffer)
2×10^{-3} M = conc. 1
OR
0.2 ml choline chloride (in buffer)
4×10^{-3} M = conc. 2

0.2 ml of 11% Potassium Ferricyanide (neutralized)

Temperature: 37.5°C.

Final Volume: 2.2 ml

Gas Phase: 1. 95% N_2: 5% CO_2
2. 95% N_2O; 5% CO_2 (Experimental)

** Enzyme prepared by the method of Bernheim and Bernheim (128) described on p. 128.
All additions from the side bulb made at 3 minutes before zero time.

TABLE XX

The Effect of Nitrous Oxide on the Choline Oxidase System of Rat Liver

	μl. of O_2 taken up/hour	
System	Air	80% N_2O: 20% O_2
Control (entire system without substrate)	6	7
Control + choline conc. 1	56*	58*
Control + choline conc. 2	88*	87*

* Corrected for blanks.

Flask Contents:

Main Compartment: 0.5 ml of enzyme**
1.3-1.8 ml of phosphate buffer (pH 7.8) depending on additions from side bulb.

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm^2

Side Bulb: 0.1 ml choline chloride (in buffer)
2×10^{-3} M = conc. 1
OR
0.2 ml choline chloride (in buffer)
4×10^{-3} M = conc. 2

Temperature: 37.5°C.

Final Volume: 2.2 ml.

Gas Phase: 1. Air
2. 80% N_2O: 20% O_2

** Enzyme prepared by the method of Bernheim and Bernheim (128) described on p. 128.
All additions from the side bulb made at zero time.

The Effect of Nitrous Oxide on Carbonic Anhydrase

The carbonic anhydrase of red blood cells is an extremely active enzyme. In view of the high solubility of nitrous oxide in blood, it was of interest to see if this gas has any effect on the activity of the enzyme.

Carbonic anhydrase was prepared from rabbit erythrocytes as follows:

> Fresh blood was collected in isotonic citrate from a rabbit and immediately centrifuged at 3,000 r.p.m. for ten minutes to pack the red cells. The supernatant plasma was removed. 1 ml of packed cells was hemolysed with 9 ml of distilled water and the cell stroma was removed by centrifugation. Aliquots of the stroma-free hemolysate (SFH) were pipetted into Warburg vessels containing cold bicarbonate (185).

The results in Table XXI and the reaction-time curves in Fig.3 show the effect produced by nitrous oxide on two concentrations of the enzyme. All the calculations are based on a 5 minute reaction period.

Data derived from Table XXI

	Percentage change in CO_2 evolution	
Time in minutes	Enzyme conc. 1	Enzyme conc. 2
1	+124	+108
3	+99	+117
5	+92	+106

TABLE XXI

The Effect of Nitrous Oxide on the Carbonic Anhydrase Activity of Rabbit Erythrocytes

	µl. of CO_2 evolved			
Time in seconds	Enzyme 1	Enzyme 1 + N_2O	Enzyme 2	Enzyme 2 + N_2O
0	0	0	0	0
30	14	28	19	45
60	26	58	39	81
90	37	83	56	112
120	47	102	70	136
150	59	120	80	171
180	68	134	88	191
210	74	149	97	202
240	81	156	104	213
270	85	164	109	225
300	89	171	112	231

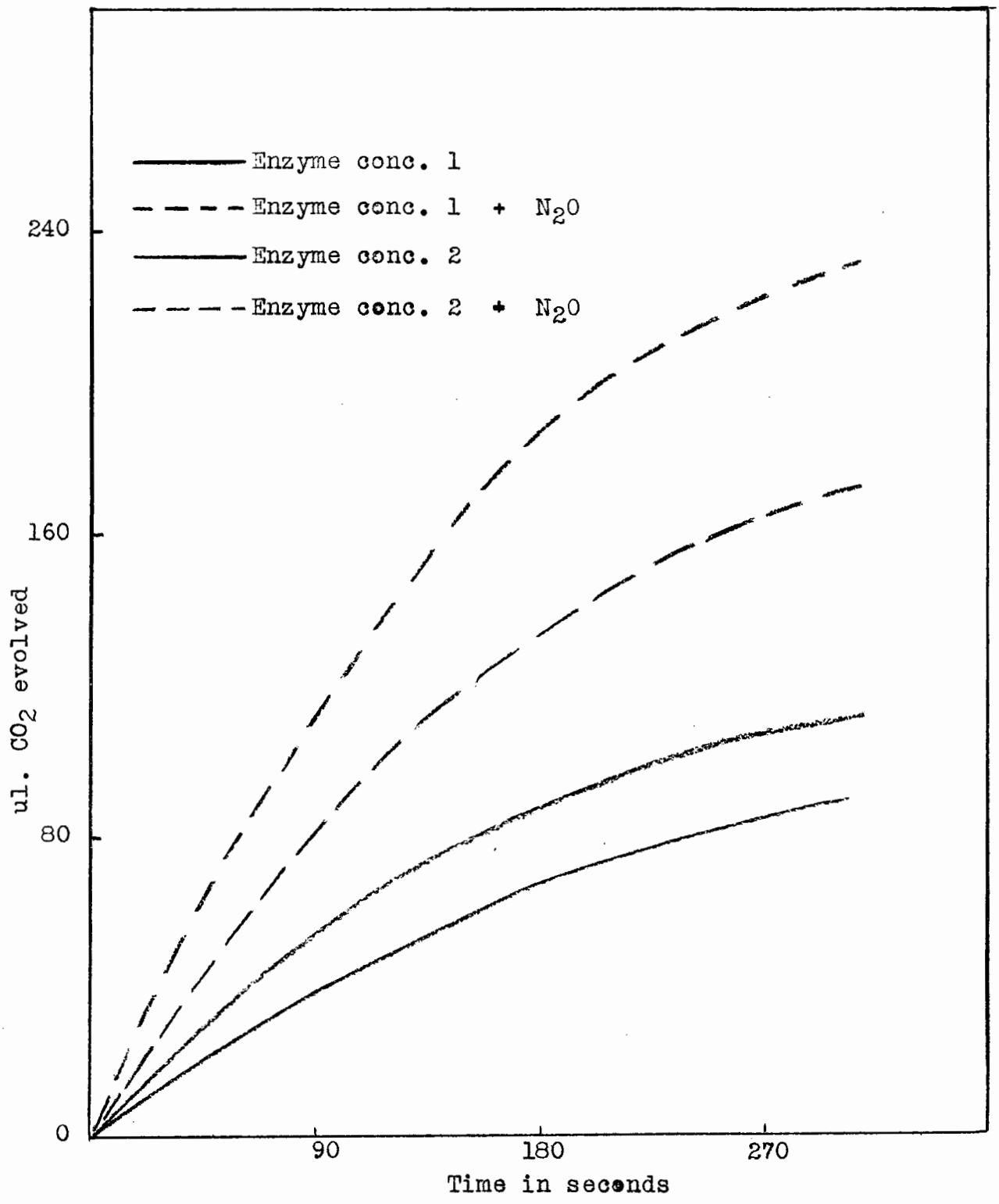
Flask Contents:
Main Compartment: 0.9 ml of 0.025 M $NaHCO_3$ in 20% glucose.*
0.1 ml of stroma-free haemolysate (Enzyme 1) or
0.2 ml of " " " (Enzyme 2)
Distilled water to make final volume to 2.2 ml.

Side Bulb: 0.9 ml of 0.5 M phosphate buffer (pH 6.6)

Temperature: 5°C. Total Volume: 2.2 ml. Gas Phase: 1. 95% N_2:5% CO_2
2. 95%N_2O:5% CO_2

Shaking Time: 135 oscillations/minute

*Note: The glucose was used in the bicarbonate solution to give the contents of the main compartment of the vessel a high density, so that when the contents of the side bulb were added there was no mixing until the shaking was begun (185).



The Effect of Nitrous Oxide on the Carbonic Anhydrase Activity of Rabbit Erythrocytes.

Fig. 3

The Effect of Nitrous Oxide on Acetylcholinesterase

The object of the following experiments was to see if N_2O has any effect on the rate of hydrolysis of acetylcholine by acetylcholinesterase. The results of these experiments are presented in Table XXII. The rate of acetylcholine hydrolysis by the enzyme is very rapid and remains the same in the presence of N_2O. A variation in the concentration of the substrate alters the rate slightly, but the effect of N_2O remains unchanged.

The Effect of Nitrous Oxide on Lactic Dehydrogenase of Yeast

Lactic dehydrogenase of yeast differs from that of muscle tissue in that it is directly linked to the cytochrome c - cytochrome oxidase system, and does not require DPN and flavoprotein as cofactors.

The enzyme was prepared as follows:

> An acetone-dried powder of yeast* was extracted with a saline-bicarbonate mixture by grinding in a mortar. The solution was made so that 1 ml of solution contained 25 mg of powder. The solution was centrifuged in the cold at 3,000 r.p.m. for 10 minutes and the clear supernatant used.

The results of these experiments are presented in Table XXIII. It can be seen that the degree of inhibition produced by nitrous oxide was not significant.

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* Kindly donated by Mr. J. Kochen, Research Institute, Montreal General Hospital, Montreal.

TABLE XXII

The Effect of Nitrous Oxide on the Hydrolysis of Acetylcholine by Acetylcholinesterase

	μl. of CO_2 evolved/hour	
System	95% N_2: 5% CO_2	95% N_2O:5% CO_2
Control (entire system without substrate)	0	7
Control + Ach conc. 1	738	710
" + Ach conc. 2	640	640
" + Ach conc. 3	590	615

Flask Contents:

Main Compartment: 1 ml brain homogenate prepared in the medium described below (100 mg. tissue)
0.8-1.2 ml of medium containing: 0.025 M $NaHCO_3$; 0.075 M KCl; 0.075 M NaCl; 0.04 M $MgCl_2$.

Side Bulb: 0.1 ml Acetylcholine chloride
4.5×10^{-3} M = Ach conc. 1 OR
0.15 ml Ach chloride, 6.8×10^{-3} M = Ach conc. 2 OR
0.2 ml Ach chloride, 9.0×10^{-3} M = Ach conc. 3

Final Volume: 2.2 ml.

Temperature: 37.5°C.

Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O:5%CO_2 (Experimental)

The rat brain homogenate was prepared in the manner described in Appendix ii.

TABLE XXIII

The Effect of Nitrous Oxide on Lactic Dehydrogenase of Yeast

System	µl. of CO_2 evolved	
	95% N_2:5% CO_2	95% N_2O:5% CO_2
Control (entire system without substrate)	128	110
Control + 0.08 M dl-lactate	522*	460*
" + 0.12 M dl-lactate	567*	526*
" + 0.16 M dl-lactate	612*	576*

* Corrected for blanks.

Flask Contents:

Main Compartment: 1 ml of yeast extract (25 mg)
1.4-1.8 mlof 0.127 M saline and
0.025 M bicarbonate mixture

Side Bulb: 0.2 ml of 0.1 M NaCN (neutralized)
0.2 ml of 11% potassium ferricyanide
0.2-0.4 ml of 1.3 M dl-lactate

Total Volume: 2.2 ml

Incubation Time: 60 minutes

Temperature: 37.5°C.

Shaking Time: 135 oscillations / minute

Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O:5% CO_2

The yeast extract was prepared by the method described on p. 134. The zero reading was taken 5 minutes after the addition of the contents of the side bulb.

DISCUSSION

The results of the experiments indicate that the choline oxidase system was not sensitive to nitrous oxide. Choline dehydrogenase, also, when studied under anaerobic conditions was not inhibited by nitrous oxide.

In the original study Mann and Quastel (130) found that choline dehydrogenase does not require DPN as an intermediate in the hydrogen transport system. However, Strength *et al.* (132) recently have made a preparation of choline dehydrogenase which was found to require DPN for optimal activity. The enzyme was prepared by the differential centrifugation of rat liver homogenate. This method differs considerably from that of Bernheim.

Our findings support the view of Mann and Quastel. The enzyme preparation used in our study did not contain DPN; nevertheless, the system was active. Other experiments already reported in this thesis have indicated that in the DPN-linked systems, the flavoprotein is sensitive to nitrous oxide. Since nitrous oxide has no effect on the choline oxidase system, it seems that flavoprotein is not involved and, hence, it may be inferred that DPN is not an essential component of the system.

As indicated in Table XXI carbonic anhydrase was not inhibited by nitrous oxide but was stimulated to twice the normal activity. The curves in Figure 3 show that the carbon dioxide evolution fell off gradually towards the end

of the five minute period. Curves of a similar type were obtained when nitrogen was replaced by nitrous oxide in the gas phase.

The possibility that a gas other than carbon dioxide was being liberated was investigated. By using KOH as an absorbent it was found that only carbon dioxide was produced by the reaction.

The effect of nitrous oxide on carbonic anhydrase is different from its effect on other enzyme systems. It is difficult to interpret the results and to explain how nitrous oxide produces the remarkable stimulation. Zinc is known to be present in carbonic anhydrase, and inhibitors of this enzyme usually act by combining with the metal. However, it seems rather unlikely that nitrous oxide, being an inert gas, can react with zinc so as to enhance the activity of the system.

Nitrous oxide produced no inhibition of the hydrolysis of acetylcholine by acetylcholinesterase.

The lactic dehydrogenase of yeast is not very sensitive to nitrous oxide. Experiments described earlier indicated that lactic dehydrogenase of animal tissue, such as brain, liver and kidney, also is only slightly sensitive to the action of nitrous oxide.

GENERAL DISCUSSION

In order to understand the mechanism of the physiological and pharmacological effects of various agents on the intact animal, it is helpful to study first the influence of the agents on the unit metabolic processes of isolated organs. The information thus obtained can then be applied in the elucidation of more generalized metabolic reactions. Such a procedure has been used in this study to ascertain the effects produced by nitrous oxide on various isolated organs and enzyme systems. The effects produced by nitrous oxide *in vivo* were explained in the light of the results obtained from *in vitro* studies.

It may be argued that an isolated tissue may not behave in the normal physiological manner when exposed to a foreign agent. True, the response of the isolated tissue may be exaggerated or the system in the abnormal condition without a blood or nerve supply may be more or less sensitive, but the character of the response is qualitatively the same as that *in vivo*.

Whether nitrous oxide itself causes anesthesia was a question often discussed in earlier years. Former authorities held the view that the narcotic effect of the gas was attributable to the hypoxia initiated by the gas rather than to its own pharmacological action. The chemical inertness of nitrous oxide and its capacity to expel oxygen from the blood specimens, as well as the cyanosis often observed during N_2O-O_2

anesthesia, appeared to support this view. However, it became apparent in later years that a state of hypoxia did not necessarily accompany the narcosis produced by mixtures of N_2O-O_2. The view nowheld by most anesthetists attributes the state of anesthesia to the direct action of nitrous oxide. However, since nitrous oxide is a weak anesthetic, a small degree of hypoxia can deepen the state of narcosis. Similarly, experiments in vitro have presented conflicting results as to the effect produced by nitrous oxide. Some have found that nitrous oxide acts by depressing certain metabolic activities of brain, whereas others claim that it produces no effect on this tissue.

The results of the experiments presented in this thesis lead to the conclusion that the inhibition observed with certain organs and enzyme systems is a result of the direct action of nitrous oxide and not merely or predominantly to a lack of oxygen. The fact that anaerobic reactions, such as glycolysis, also were inhibited further confirms this view. In all our experiments the gas mixtures contained 80% nitrous oxide which is equivalent to the proportion of nitrogen in air. The oxygen content also was the same as in air.

The human patient as well as the experimental animal, does not show any ill-effects under N_2O-O_2 anesthesia, as long as ample oxygen is present. However, it is difficult to say, whether it would be possible to keep an animal in such a con-

dition for several hours without causing serious metabolic disturbances. Since certain metabolic reactions are arrested by the action of nitrous oxide it is likely that the inhibition of the functions for a prolonged period would be injurious.

The margin of N_2O-O_2 anesthesia is very narrow. Under the optimum mixture of N_2O-O_2 the patient may be kept in a controlled state of anesthesia. But a small increase in the proportion of oxygen may cause him to awaken, or a decrease in the proportion may produce a hypoxic condition.

Cyanosis during nitrous oxide anesthesia need not give cause for alarm. The presence of 5 gms.% of reduced hemoglobin in the blood is sufficient to produce visible cyanosis. This does not necessarily mean that the patient is suffering from a lack of oxygen. Anesthetists, experienced in the administration of N_2O-O_2 anesthesia, know from long clinical experience with nitrous oxide that the blood contains ample oxygen notwithstanding the cyanosis. True hypoxia as reflected by the irregular breathing of the patient, on the contrary, is serious and must be avoided.

The recovery from N_2O-O_2 anesthesia is usually rapid and uneventful, and represents one of the great advantages of the use of nitrous oxide. The rapid recovery of normal enzyme activity has also been observed in experiments with isolated tissues. Simply replacing the nitrous oxide in the gas phase by nitrogen completely reverses any inhibition. This implies that the energy metabolism of the cell is not damaged during

exposure to nitrous oxide even though certain enzyme systems may have been inhibited.

A narcotic has been defined as "any drug that produces sleep or a stupor and at the same time relieves pain". Since this definition is applicable to nitrous oxide the gas may be considered to be a narcotic. The tissue oxidation theory proposed by Quastel and his associates to explain the mechanism of narcosis can be applied partly to the action of nitrous oxide. Nitrous oxide as well as certain other narcotics such as barbiturates, chloretone, etc., studied by Quastel, produces a marked inhibition of tissue respiration. However, the point of inhibition in the respiratory chain due to nitrous oxide is not the same as with other narcotics. Quastel found that a factor situated between flavoprotein and the cytochrome c in the system is inhibited. Studies with nitrous oxide, however, have shown that the flavoprotein itself is sensitive to the gas.

The oxidation of succinate is not affected by a number of drugs studied by Quastel. Nitrous oxide, however, inhibits succinate oxidation, and the sensitive factor in the system appears to be cytochrome b.

The experiments of Quastel have shown that several dehydrogenases are not sensitive to narcotics. It has been found in the present study that lactic dehydrogenase of yeast is not N_2O-sensitive, but that lactic dehydrogenase, glucose dehydrogenase and iso-citric dehydrogenase of animal tissues

are slightly sensitive.

Chloretone and certain other narcotics do not inhibit glycolysis in concentrations that produce a significant decrease in the oxygen consumption. The system is strongly inhibited, however, by nitrous oxide.

The mode of action of various agents on enzyme systems differs with the agent. Some inhibitors react with active sites on the enzyme. Many SH-enzymes are inactivated in that way. Other inhibitors compete with the substrate for the enzyme. The inhibition may be reversible or irreversible. The majority of narcotics, if used in moderate concentrations, produce a reversible type of inhibition. However, very high concentrations may cause irreversible inhibition thus indicating damage to the enzyme. This type of inhibition and damage may occur also if the inhibitor reacts chemically with the enzyme, for example the inhibition of acetylcholinesterase by tetraethyl pyrophosphate.

How nitrous oxide inhibits certain enzyme systems is not yet understood. Nitrous oxide is a chemically inert gas and therefore does not react chemically with other compounds. The fact that the inhibition is so readily reversible indicates that, if any combination between the gas and the enzyme can occur, it must be a very loose complex.

Enzymes whose reactive site is known to be the sulfhydryl group are not inhibited by nitrous oxide. Succinic dehydrogenase for example, is insensitive to the gas. Cytochrome b

is the only nitrous oxide-sensitive member of the cytochrome group. The inhibition probably does not involve the iron of the molecule since if this were so the other cytochromes should also be inhibited by nitrous oxide.

Some drugs inhibit nearly all enzyme systems. Benzimidazole is an example of this type of drug. Certain other agents including nitrous oxide inhibit only certain enzymes. Among the enzymes not sensitive to this gas is acetylcholinesterase. Acetylcholine has been called the vagus hormone and is supposed to be responsible for the excitation of the nerve. Acetylcholine and acetylcholinesterase are believed to be involved in the transmission of nerve impulses. The fact that nitrous oxide does not inhibit the hydrolysis of acetylcholine implies that neural transmission is not impaired in the presence of the gas.

The work of Featherstone and Levy has shown that nitrous oxide does not inhibit oxidative phosphorylation. This view is consistent with the observed rapid and complete recovery from the effects of nitrous oxide. Since the aerobic synthesis of ATP is not affected by nitrous oxide it is evident that the energy-capturing mechanism of the cell is unimpaired. Narcotics, such as the barbiturates, which inhibit oxidative phosphorylation, cause a decrease in the energy stores of the body, and if administered for a very long period cause irreparable damage.

Nitrous oxide when used in combination with other drugs

may have more serious effects than those so far described. Since the use of nitrous oxide together with curare and pentothal sodium is becoming fairly common in surgery, it would be of importance to ascertain the effects produced by the combined use of nitrous oxide and narcotics. These questions will be studied further in connection with the current investigation.

SUMMARY

1. The effect of nitrous oxide in the rat was studied, in *in vivo* and *in vitro* experiments. It was found that a 4:1 mixture of N_2O keeps the animal anesthetized for a long period of time, without causing any deleterious effect on the animal.
2. Pure nitrous oxide when passed through rat blood *in vitro* expelled all the oxygen from the blood. On reexposure of the samples to oxygen, the hemoglobin was rapidly converted to oxyhemoglobin. A mixture of N_2O-O_2 produced no observable change when bubbled through the blood.
3. The respiration of brain slices and homogenates with glucose, lactate, pyruvate, iso-citrate and succinate was found to be inhibited by N_2O. The degree of inhibition was much lower with homogenates than with slices.
4. The anaerobic metabolism of brain tissue is slightly inhibited by N_2O.
5. The respiration of liver and kidney tissue was found to be inhibited by N_2O to the same extent as with brain tissue. Again the inhibition with homogenates was much lower than that with slices, except in the case of succinate where both slices and homogenates showed the same degree of inhibition.

6. The inhibition of the anaerobic metabolism of liver tissue was too low to be considered significant. The inhibition with kidney tissue was slightly higher.
7. The addition of DPN to rat brain homogenate did not alter the degree of inhibition by N_2O.
8. The N_2O-sensitive components of the respiratory chain were found to be cytochrome b and the flavoprotein.
9. Succinic dehydrogenase is not sensitive to N_2O.
10. The lactic and iso-citric dehydrogenases of brain and kidney tissue are slightly inhibited by N_2O. The same enzymes in liver are not N_2O-sensitive.
11. The oxidation of pyruvate by brain, liver and kidney is inhibited by N_2O.
12. Nitrous oxide was shown to inhibit the anaerobic glycolysis of rat brain extracts. The addition of increasing amounts of ATP did not alter the degree of inhibition, thus showing that the system did not fail from lack of ATP.
13. The inhibition produced by N_2O was the same when glucose, glucose-6-phosphate, fructose-6-phosphate, or fructose-1, 6-diphosphate was used as substrate.
14. The anaerobic acetylation of sulfanilamide by pigeon liver extracts in the presence of acetate and ATP was not inhibited by N_2O.

15. The aerobic acetylation of sulfanilamide by pigeon liver extracts and rat brain homogenates was inhibited slightly by N_2O in the presence of acetate and ATP.
16. The aerobic acetylation of sulfanilamide resulting from the dismutation of pyruvate was sensitive to N_2O. The addition of DPN did not alter the inhibition. The addition of ATP alone or together with DPN caused a slight decrease in the degree of inhibition.
17. The choline dehydrogenase and choline oxidase systems were found to be insensitive to N_2O. This supports the findings of Mann and Quastel who state that choline dehydrogenase is linked directly to cytochrome c and does not require DPN.
18. It was found that carbonic anhydrase of rabbit erythrocytes was stimulated to double its activity by N_2O.
19. The activity of acetylcholinesterase in hydrolyzing acetylcholine is not sensitive to N_2O.
20. Nitrous oxide does not significantly inhibit lactic dehydrogenase of yeast.
21. The recovery from the action of N_2O on both the respiration of liver and kidney, and the glycolysis of brain was found to be rapid and complete.

APPENDIX

( ) Indicates controls without added substrate

Appendix i

Data for Table I, p.77

The Effect of Nitrous Oxide on the Respiration of Rat Brain Cortex Slices

System	Average $-Q_{O_2}$ Values				
	No Substrate Added	Succinate	Lactate	Pyruvate	Iso-Citrate
Control (Air)	7.8	(7.8)	(7.8)	(6.0)	(6.2)
" + Substrate (Air)	-	7.6	11.7	7.4	7.7
" + N_2O	3.7	(3.7)	(3.7)	(3.1)	(3.2)
" + Substrate + N_2O	-	3.9	5.8	2.6	3.1

Flask Contents:
Main Compartment: Cold Krebs-Ringer-Phosphate-Glucose (Ca free) 1.8-2.0 ml. (depending on additions from side bulb)

Center Well: 0.2 ml. 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml. of substrate 0.01 M final concentration

Temperature: 37.5°C. Final Volume: 2.2 ml. Gas Phase: 1. - Air. 2. - 80% N_2O:20% O_2

(Experimental) Tissue Dry Weight: 1/5 of humid-cut weight. Shaking Time: 135 oscillations/minute

Humid-cut rat brain cortex slices were suspended in Warburg vessels containing cold KRPG medium and gassed (experimental flasks only) for 10 minutes. After temperature equilibration the contents of the side bulb were added to the tissue slice and the $-Q_{O_2}$ values obtained in the following 60 minutes are shown in the Table above.

Appendix ii

Data for Table I, p.77

The Effect of Nitrous Oxide on the Respiration of Rat Brain Homogenate

System	Average ul. Oxygen taken up/hour				
	No Substrate Added	Succinate	Lactate	Pyruvate	Iso-Citrate
Control (Air)	110	(145)	(110)	(106)	(110)
" + Substrate (Air)	-	257	142	158	150
" + N_2O	100	(140)	(100)	(100)	(100)
" + Substrate + N_2O	-	211	110	100	126

Flask Contents:
Main Compartment: 1 ml. homogenized rat brain (100 mgs.) Cold Krebs-Ringer-Phosphate-Glucose (Ca free with 0.017 M Nicotinamide) 0.6-1.0 depending on the additions from the side bulb.

Center Well: 0.2 ml. of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml. of 0.1 M substrate. Temperature: 37.5°C. Final Volume: 2.2 ml.

Gas Phase: 1. - Air. 2. - 80% N_2O:20% O_2 (Experimental). Shaking Time: 135 oscillations/minute.

Freshly dissected rat brain was homogenized in cold KRPG containing 0.017 M nicotinamide, to give a final tissue concentration of 100/ml. of homogenate. 1 ml. of homogenate was introduced into Warburg vessels containing cold KRPG. The experimental flasks were gassed for 10 minutes. After temperature equilibration, the contents of the side bulb were added and the respiration for the subsequent 60 minutes is shown in the Table above.

Appendix iii

Data for Table II, p. 83

The Effect of Nitrous Oxide on the Respiration of Rat Brain Cortex Slices in the Presence of Methylene Blue

System	Average $-Q_{O_2}$ Values				
	No Substrate Added	Succinate	Lactate	Pyruvate	Iso-Citrate
Control (Air)	9.6	(8.4)	(8.9)	(8.9)	(9.2)
" + Substrate (Air)	-	12.2	8.8	11.6	9.6
" + N_2O	3.2	(2.2)	(2.4)	(2.4)	(2.6)
" + Substrate + N_2O	-	5.4	4.0	2.0	3.8

Flask Contents:
Main Compartment: Cold Krebs-Ringer-Phosphate-Glucose (Ca free). 1.6-1.8 ml. (depending on additions from side bulb).

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml. of 0.1 M Substrate. 0.2 ml. Methylene Blue (1×10^{-5} M).

Temperature: 37.5°C. Final Volume: 2.2 ml. Gas Phase: 1. -Air. -80% N_2O:20% O_2 (Experimental)

Shaking Time: 135 oscillations/minute. Tissue Dry Weight: 1/5 of humid-cut weight.

Humid-cut rat brain cortex slices were suspended in Warburg vessels containing cold KRPG medium and gassed (experimental flasks only) for 10 minutes. After temperature equilibration the contents of the side bulb were added to the tissue slice and the $-Q_{O_2}$ values obtained in the following 60 minutes are shown in the Table above.

Appendix iv

Data for Table II, p. 83

The Effect of Nitrous Oxide on the Respiration of Rat Brain Homogenate in the Presence of Methylene Blue

System	Average ul of Oxygen taken up/hour				
	No Substrate Added	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	210	(238)	(207)	(205)	(205)
" + Substrate (Air)	-	380	272	307	180
" + N_2O	166	(188)	(146)	(147)	(147)
" + Substrate + N_2O	-	332	192	189	152

Flask Contents:
Main Compartment: 1 ml homogenized rat brain (100 mg.)
0.6-1.0 ml cold KRPG (Ca free with 0.017 M nicotinamide)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate; 0.2 ml of 1×10^{-5} M methylene blue

Temperature: 37.5°C. Final Volume: 2.2 ml Shaking Time: 135 oscillations/minute

Gas Phase: 1. Air. 2. 80% N_2O: 20% O_2

The homogenate was prepared by the method described in Appendix ii.

Appendix v

Data for Table III, p. 87

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Brain Cortex Slices. (Ferricyanide Technique.)

System	No Substrate Added	Average Q_{CO_2} Values Succinate	Lactate	Pyruvate	iso-Citrate
Control	5.6	(5.6)	(6.7)	(5.3)	(5.3)
" + Substrate	-	17.7	6.4	9.9	6.6
" + N_2O	4.5	(4.5)	(4.2)	(5.2)	(5.5)
" + Substrate + N_2O	-	18.5	5.2	7.1	4.9

Flask Contents:
Main Compartment: 1.6-2.0 ml Cold KRBG depending on the additions from the side bulb

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide (neutralized)
0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml. Shaking Time: 135 oscillations/minute

Gas Phase: 1. 95% N_2: 5% CO_2
2. 95% N_2O: 5% CO_2

Tissue Dry Weight: 1/5 of humid-cut weight

Humid-cut rat brain cortex slices were suspended in Warburg vessels containing cold KRBG and gassed for 10 minutes. After temperature equilibration, the contents of the side bulb were added to the tissue slice in the main compartment and the first reading was taken 5 minutes later. The results shown in the above table are the Q_{CO_2} values for the subsequent 60 minutes.

Appendix vi

Data for Table III, p. 87

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Brain Homogenates. (Ferricyanide Technique.)

System	Average µl of CO_2 evolved/hour				
	No Substrate Added	Succinate	Lactate	Pyruvate	iso-Citrate
Control	125	(152)	(125)	(148)	(130)
" + Substrate	-	550	124	215	137
" + N_2O	106	(106)	(106)	(118)	(107)
" + Substrate + N_2O	-	600	107	137	112

Flask Contents:
Main Compartment: 0.8-1.0 ml Cold KRBG (with 0.017 M Nicotinamide)
1.0 ml homogenized rat brain (100 mg)

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide (neutralized)
0.2 ml of 0.1 M substrate

Total Volume: 2.2 ml. Temperature: 37.5°C. Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O:5% CO_2

Shaking Time: 135 oscillations/minute.

Freshly dissected rat brain was homogenized in cold KRBG (containing 0.017 M nicotinamide) in a volume that gives a final tissue concentration of 100 mg./ml. of homogenate. 1 ml of homogenate was introduced into Warburg vessels containing cold KRBG and the vessels were gassed for 10 minutes. After temperature equilibration, the contents of the side bulb were added to the main compartment and the first reading was taken 5 minutes later. The results shown in the above table represent the CO_2 evolved in the subsequent 60 minutes.

Appendix vii

Data for Table IV, p. 91

The Effect of Nitrous Oxide on the Respiration of Rat Brain Homogenate in the Presence of Added DPN

System	Average µl. of Oxygen taken up	
	Air	80% N_2O:20% O_2/hour
Control	93	83
" + Pyruvate	100	69
" + " + DPN	122	75

Flask Contents:

Main Compartment: 1.6-1.8 ml of medium containing: 0.03 M KCl; 0.03 M $MgCl_2$; 0.05 M NaF; 0.005 M nicotinamide; 0.01 M phosphate buffer (pH 7.5)
1.0 ml of rat brain homogenate

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml pyruvate 0.02 M
0.2 ml DPN 4.3×10^{-4} M

Temperature: 37.5°C.

Final Volume: 3.2 ml

Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Shaking Time: 135 oscillations/minute

Freshly dissected rat brain was homogenized in cold medium in a volume that gives a final tissue concentration of 100 mg. per ml of homogenate. 1 ml. of homogenate was introduced into Warburg vessels containing cold medium. The experimental flasks were gassed for 10 minutes. After temperature equilibration, the contents of the side bulb were added and the respiration for the subsequent 60 minutes is shown in the above table.

Appendix viii

Data for Table V, p. 92

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Brain Homogenate in the Presence of Added DPN

(Ferricyanide Technique)

System	Average µl. of CO_2 evolved/hour		
	No Added Substrate	Lactate	Pyruvate
Control	95	(95)	(95)
" + DPN	165	(165)	(165)
" + Substrate	-	131	128
" + " + DPN	-	178	162
" + N_2O	71	(71)	(71)
" + DPN + N_2O	116	(116)	(116)
" + Substrate + N_2O	-	88	88
" + " + DPN + N_2O	-	133	120

Flask Contents:
Main Compartment: 0.6-1.0 ml Cold KRBG (with 0.017 M nicotinamide)
1.0 ml homogenized brain (100 mg)

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide
0.2 ml of 0.1 M substrate
0.2 ml of DPN (5.4×10^{-4} M final conc.)

Temperature: 37.5°C. Final Volume: 2.2 ml

Shaking Time: 135 oscillations/minute Gas Phase: 1. 95%N_2:5%CO_2
2. 95%N_2O:5%CO_2 (Experimental)

The rat brain homogenate was prepared as described in Appendix vi. The zero reading was taken 5 minutes after adding the contents of the side bulb.

Appendix ix

Data for Table VI, p. 94

The Effect of Nitrous Oxide on the Respiration of Rat Liver Slices

System	Average $-Q_{O_2}$ Values				
	No Substrate Added	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	4.5	(5.4)	(3.5)	(4.8)	(4.0)
" + Substrate (Air)	-	11.2	4.2	5.4	4.1
" + N_2O	1.4	(2.9)	(1.9)	(2.3)	(1.4)
" + Substrate + N_2O	-	8.3	1.8	1.5	1.6

Flask Contents:
Main Compartment: 1.6-1.8 ml Cold KRPG (Ca free)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml Gas Phase: 1. Air
2. 80% N_2O: 20% O_2 (Experimental)

Tissue Dry Weight: 1/3.7 of humid-cut weight.

Humid-cut rat liver slices were suspended in Warburg vessels containing cold KRPG medium and gassed (experimental flasks only) for 10 minutes. After temperature equilibration, the contents of the side bulb were added to the tissue slice in the main compartment and the zero reading was taken immediately. The values obtained in the following 60 minutes are shown in the table above.

Appendix x

Data for Table VII, p. 96

The Effect of Nitrous Oxide on the Respiration of Rat Kidney Cortex Slices

System	Average $-Q_{O_2}$ Values				
	No Substrate Added	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	5.3	(5.3)	(5.9)	(5.6)	(6.0)
" + Substrate (Air)	-	6.3	6.1	5.7	6.2
" + N_2O	1.8	(1.8)	(1.3)	(1.2)	(1.9)
" + Substrate + N_2O	-	3.7	2.1	2.6	1.8

Flask Contents:
Main Compartment: 1.6-1.8 ml Cold KRPG

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of o.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml. Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Tissue Dry Weight: ¼ of humid-cut weight

Humid-cut rat kidney cortex slices were suspended in Warburg vessels containing cold KRPG medium and gassed (experimental flasks only) for 10 minutes. After temperature equilibration, the contents of the side bulb were added to the tissue slice and the $-Q_{O_2}$ values obtained in the following 60 minutes are shown in the table above.

Appendix xi

Data for Table VI, p.94

The Effect of Nitrous Oxide on the Respiration of Rat Liver Homogenates

System	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
	Average µl. of Oxygen taken up / hour				
Control (Air)	63	(54)	(72)	(72)	(166)
" + Substrate (Air)	-	454	110	72	184
" + N_2O	58	(58)	(58)	(58)	(180)
" + Substrate + N_2O	-	274	100	71	186

Flask Contents:
Main Compartment: 0.6-1.0 ml Cold KRPG (Ca free with 0.017 M Nicotinamide)
1.0 ml homogenized rat liver (100 mg.)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate

Temperature: 37.5°C° Final Volume: 2.2 ml. Gas Phase: 1. Air
2. 80% N_2O:20% O_2 (Experimental)

Freshly dissected rat liver was homogenized in cold KRPG containing 0.017 M nicotinamide, to give a final tissue concentration of 100 mg/ ml of homogenate. 1 ml of homogenate was introduced into Warburg vessels containing cold KRPG. The experimental flasks were gassed for 10 minutes. After temperature equilibration, the contents of the side bulb were added and the respiration for the subsequent 60 minutes is shown in the table above.

Appendix xii

Data for Table VII, p. 96

The Effect of Nitrous Oxide on the Respiration of Rat Kidney Homogenate

System	Average µl. of Oxygen taken up / hour				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	72	(74)	(121)	(70)	(174)
" + Substrate (Air)	-	500	138	120	169
" + N_2O	67	(80)	(102)	(55)	(168)
" + Substrate + N_2O	-	274	127	106	172

Flask Contents:
Main Compartment: 0.6-1.0 ml Cold KRPG (Ca free with 0.017 M nicotinamide)
1.0 ml homogenized rat kidney (100 mg.)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Rat Kidney homogenate prepared in the manner as described for liver homogenate in Appendix xi.

Appendix xiii

Data for Table VIII, p. 99

The Effect of Nitrous Oxide on the Respiration of Rat Liver Slices in the Presence of Methylene Blue

System	Average $-Q_{O_2}$ Values				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	3.6	(3.6)	(4.0)	(3.4)	(4.1)
" + Substrate (Air)	-	8.5	4.6	4.5	4.2
" + N_2O	1.4	(1.4)	(1.6)	(2.6)	(1.6)
" + Substrate + N_2O	-	4.1	2.2	1.9	1.5

Flask Contents:
Main Compartment: 1.6-1.8 ml Cold KRPG (Ca free)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate
0.2 ml of 1×10^{-5} M methylene blue

Temperature: 37.5°C. Final Volume: 2.2 ml. Shaking Time: 135 oscillations/minute

Tissue Dry Weight: 1/3.7 of humid-cut weight Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Liver slices prepared in the manner described in Appendi ix. The zero reading was taken immediately after adding the contents of the side bulb.

Appendix xiv

Data for Table IX, p. 101

The Effect of Nitrous Oxide on the Respiration of Rat Kidney Cortex Slices in the Presence of Methylene Blue

System	Average $-Q_{O_2}$ Values				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	7.4	(7.4)	(7.4)	(9.2)	(6.5)
" + Substrate	-	8.1	7.9	10.9	6.5
" + N_2O	2.8	(2.8)	(2.8)	(2.3)	(1.6)
" + Substrate + N_2O	-	4.2	3.1	3.2	1.8

Flask Contents:
Main Compartment: 1.6-1.8 ml Cold KRPG (Ca free)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of substrate 0.1 M
0.2 ml of 1 x 10^{-5} M methylene blue

Temperature: 37.5°C. Final Volume: 2.2 ml. Shaking Time: 135 oscillations/minute

Tissue Dry Weight: 1/4 of humid-cut weight Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Kidney cortex slices prepared in the manner described in Appendix x. The zero reading was taken immediately after adding the contents of the side bulb.

Appendix xv

Data for Table VIII, p. 99

The Effect of Nitrous Oxide on the Respiration of Rat Liver Homogenates in the Presence of Methylene Blue

	Average µl. of Oxygen taken up/hour				
System	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	54	(54)	(54)	(73)	(41)
" + Substrate (Air)	-	390	70	95	65
" + N_2O	56	(56)	(56)	(70)	(33)
" + Substrate + N_2O	-	256	76	93	57

Flask Contents:
Main Compartment: 0.6-1.0 ml Cold KRPG (Ca free with 0.017 M nicotinamide)
1.0 ml homogenized rat liver (100 mgs.)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate
0.2 ml of 1×10^{-5} M methylene blue

Temperature: 37.5°C. Final Volume: 2.2 ml. Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Shaking Time: 135 oscillations/minute

Rat liver homogenate prepared in the manner described in Appendix xi. The zero reading was taken immediately after adding the contents of the side bulb.

Appendix xvi

Data for Table IX, p. 101

The Effect of Nitrous Oxide on the Respiration of Rat Kidney Homogenate in the Presence of Methylene Blue

System	Average µl. of Oxygen taken up/hour				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control (Air)	118	(118)	(58)	(71)	(71)
" + Substrate (Air)	-	514	114	75	70
" + N_2O	106	(106)	(60)	(58)	(58)
" + Substrate + N_2O	-	280	120	74	71

Flask Contents:
Main Compartment: 0.6-1.0 ml Cold KRPG (Ca free with 0.017 M nicotinamide)
1.0 ml of homogenized rat kidney (100 mg.)

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Side Bulb: 0.2 ml of 0.1 M substrate
0.2 ml of 1 x 10^{-5} M methylene blue

Temperature: 37.5°C. Final Volume: 2.2 ml. Gas Phase: 1. Air
2. 80% N_2O:20% O_2

Shaking Time: 135 oscillations/minute

Rat kidney homogenate prepared as described for liver homogenate in Appendix xi. The zero reading was taken immediately after adding the contents of the side bulb.

Appendix xvii

Data for Table X, p. 103

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Liver Slices

(Ferricyanide Technique)

System	Average Q_{CO_2} Values				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control	7.6	(7.1)	(6.8)	(7.6)	(7.6)
" + Substrate	-	14.9	7.5	9.7	8.4
" + N_2O	6.9	(7.7)	(5.8)	(6.9)	(6.9)
" + Substrate + N_2O	-	15.4	6.9	9.1	7.2

Flask Contents:
Main Compartment: 1.8-2.0 ml Cold KRBG depending on additions from side bulb

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide (neutralized)
0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml Tissue Dry Weight: 1/3.7 of humid-cut weight

Shaking Time: 135 oscillations/minute Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O:5% CO_2 (Experimental)

The liver slices were prepared in the manner described in Appendix v for brain cortex slices. The zero reading was taken 5 minutes after adding the contents of the side bulb.

Appendix xviii

Data for Table X, p.103

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Liver Homogenate

(Ferricyanide Technique)

System	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
	Average µl. of CO_2 evolved/hour				
Control	165	(165)	(149)	(192)	(147)
" + Substrate	-	570	190	208	152
" + N_2O	145	(145)	(140)	(155)	(114)
" + Substrate + N_2O	-	590	165	190	128

Flask Contents:
Main Compartment: 0.8-1.0 ml Cold KRBG (with 0.017 M nicotinamide)
1.0 ml homogenized rat liver (100 mg.)

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide (neutralized)
0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml Shaking Time: 135 oscillations/minute

Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O: 5% CO_2 (Experimental)

The liver homogenate was prepared in the same manner as described for brain homogenate in Appendix vi. The zero reading was taken 5 minutes after adding the contents of the side bulb.

Appendix xix

Data for Table XI, p. 105

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Kidney Cortex Slices. (Ferricyanide Technique)

System	Average Q_{CO_2} Values				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control	9.8	(8.0)	(9.9)	(11.0)	(10.5)
" + Substrate	-	15.0	13.7	14.3	10.3
" + N_2O	8.1	(7.6)	(9.6)	(9.6)	(10.3)
" + Substrate + N_2O	-	14.0	11.9	9.4	7.8

Flask Contents:
Main Compartment: 1.6-2.0 ml Cold KRBG depending on additions from the side bulb

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide (neutralized)
0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml Tissue Dry Weight: ¼ of humid-cut weight

Shaking Time: 135 oscillations/minute Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O:5% CO_2

The kidney cortex slices were prepared in the same manner as described for brain slices in Appendix v. The zero reading was taken 5 minutes after adding the contents of the bulb.

Appendix xx

Data for Table XI, p.105

The Effect of Nitrous Oxide on the Anaerobic Metabolism of Rat Kidney Homogenate

(Ferricyanide Technique)

System	Average µl. of CO_2 evolved/hour				
	No Added Substrate	Succinate	Lactate	Pyruvate	iso-Citrate
Control	142	(142)	(110)	(125)	(168)
" + Substrate	-	610	178	165	178
" + N_2O	115	(115)	(90)	(100)	(128)
" + Substrate + N_2O	-	615	135	120	151

Flask Contents:
Main Compartment: 0.8-1.0 ml Cold KRBG (with 0.017 M nicotinamide)
1.0 ml homogenized rat kidney (100 mg.)

Side Bulb: 0.2 ml of 11% Potassium Ferricyanide (neutralized)
0.2 ml of 0.1 M substrate

Temperature: 37.5°C. Final Volume: 2.2 ml. Shaking Time: 135 oscillations/minute

Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O: 5% CO_2 (Experimental)

The rat kidney homogenate was prepared in the same manner as described for brain homogenate in Appendix vi. The zero reading was taken 5 minutes after the addition of the contents of the side bulb.

Appendix xxi

Data for Table XIII, p. 111

The Effect of Nitrous Oxide on the Anaerobic Glycolysis of Rat Brain Extracts Using Various Concentrations of ATP

	μl. of CO_2 evolved/30 minutes	
System	95% N_2:5% CO_2	95% N_2O:5% CO_2
Control + Glucose (No ATP)	27	8
" + " + ATP_1	120	35
" + " + ATP_2	270	58
" + " + ATP_3	353	108

All values corrected for blanks

Flask Contents:
Main Compartment: 1.2-0.9 ml of medium containing: 0.07 M NaCl; 0.08 M $NaHCO_3$; 0.003 M $MgSO_4$; 0.0035 M KCl; 0.017 M nicotinamide,
0.2 ml DPN 4.5×10^{-4} M final conc.
0.4 ml rat brain extract = 100 mg. tissue

Side Bulb: 0.1 ml of 1.4×10^{-2}M HDP 0.1 ml of 0.1 M glucose
0.2 ml of 0.1 M phosphate buffer (pH 7.4)
0.1-0.3 ml of 0.007 M ATP. $ATP_1 = 3 \times 10^{-4}$ M
$ATP_2 = 6 \times 10^{-4}$ M
$ATP_3 = 10 \times 10^{-4}$ M

Temperature: 37.5°C. Final Volume: 2.2 ml. Gas Phase: 1. 95%N_2:5%CO_2
2. 95%N_2O:5%CO_2

The rat brain was homogenized in the medium described above. The homogenate was centrifuged in the cold at 3,000 r.p.m. for 10 minutes. The supernatant was made up to a final volume so that each ml contained the equivalent of 250 mg. of tissue. 0.4 ml of extract was pipetted into the Warburg vessels. The manometers were gassed for 10 minutes. After temperature equilibration, the contents of the side bulb were added to the main compartment, and the first reading was taken immediately. The values for the subsequent 30 minutes are shown above.

Appendix xxii

Data for Table XIV, p. 112

The Effect of Nitrous Oxide on the Anaerobic Glycolysis of Rat Brain Extracts Using Various Substrates

	µl. of CO_2 evolved/30 minutes	
System	95% N_2:5% CO_2	95% N_2O:5% CO_2
Control (Contains ATP)	73	23
" + 10 mM Glucose	160	45
" + 20 mM Glucose-6-Phosphate	230	60
" + 20 mM Fructose-6-Phosphate	220	56
" + 20 mM Fructose-1,6-diphosphate	142	38

All values corrected for blanks.

Flask Contents:

Main Compartment: 1.1 ml of medium described in Appendix xxi
0.2 ml DPN 4.5×10^{-4} M final conc.
0.4 ml rat brain extract = 100 mg tissue

Side Bulb: 0.2 ml of phosphate buffer pH 7.4
0.1 ml of 0.03 M ATP
0.2 ml of substrate

Temperature: 37.5°C.

Final Volume: 2.2 ml

Gas Phase: 1. 95% N_2:5% CO_2
2. 95% N_2O:5% CO_2

The rat brain extract was prepared in the manner described in Appendix xxi. The zero reading was taken immediately after adding the contents of the side bulb. The values for the subsequent 30 minutes are shown above.

Appendix xxiii

Data for Table XVII, p. 122

The Effect of Nitrous Oxide on the Respiration and Acetylation of Rat Brain Homogenate and Pigeon Liver Extract in the Presence of Acetate

System	Air		80% N_2O:20% O_2	
	µl. O_2 taken up / hour	µg. sulfanilamide acetylated/hour	µl. O_2 taken up / hour	µg. sulfanilamide acetylated/hour
Control	169	45	139	38
" + Acetate	209	48	169	40
" + " + ATP	240	71	220	67

All values corrected for blanks.

Flask Contents:

Main Compartment: 0.8 ml rat brain homogenate (200 mg.); 0.7 ml pigeon liver extract (90 mg powder); 0.4 ml of 0.08 M phosphate buffer (pH 7.5); Cysteine 0.01M final conc.; 0.2 ml sulfanilamide (105 µg); 0.2 ml $MgCl_2$ 0.03 M final conc; 0-0.7 ml of 0.154 M NaCl

Side Bulb: 0.2 ml of acetate 0.02 M final conc.; 0.3 ml of 0.03 M ATP

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Temperature: 37.5°C. Final Volume: 3.2 ml. Gas Phase: 1. Air 2. 80% N_2O:20% O_2 (Experimental)

A 25% rat brain homogenate was prepared in a medium containing: 0.03M KCl; 0.005M nicotinamide 0.05M NaF. 0.8 ml of homogenate was pipetted into the Warburg vessels. 0.7 ml of pigeon liver extract prepared in the same medium by the method described on p.65 was pipetted into the vessels. The experimental flasks were gassed for 10 minutes during the equilibration period. The zero reading was taken 5 minutes later and the values for the subsequent 60 minutes are shown above.

Appendix xxiv

Data for Table XVIII, p. 123

The Effect of Nitrous Oxide on the Respiration and Acetylation of Rat Brain Homogenate and Pigeon Liver Extract in the Presence of Pyruvate

System	Air		80% N_2O:20% O_2	
	µl. O_2 taken up/hour	µg. sulfanilamide acetylated/hour	µl. O_2 taken up/hour	µg. sulfanilamide acetylated/hour
Control	196	32	167	22
" + Pyruvate	210	35	139	16
" + " + DPN	244	37	150	18
" + " + ATP	311	63	176	40
" + " + DPN + ATP	281	78	160	45

All values corrected for blanks.

Flask Contents:

Main Compartment: 0.8 ml rat brain homogenate (200 mg.); 0.7 ml pigeon liver extract (90 mg. powder); 0.4 ml 0.08 M phosphate buffer (pH 7.5); Cysteine 0.01 M final conc.; 0.2 ml sulfanilamide (105 µg.); 0.2 ml $MgCl_2$ 0.03 M final conc.; 0-0.7 ml of 0.154 M NaCl

Side Bulb: 0.2 ml pyruvate 0.02 M final conc.; 0.2 ml DPN 4.3×10^{-4} M final conc.; 0.3 ml of 0.03 M ATP

Center Well: 0.2 ml of 20% KOH and filter paper 35 mm square

Temperature: 37.5°C. Final Volume: 3.2 ml. Gas Phase: 1. Air 2. 80% N_2O:20% O_2 (Experimental)

The contents of the flask were prepared in the manner described in Appendix xxiii.

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