paper.[6] Total and acetone-soluble materials were even higher in Whatman No. 3 paper. These substances are particularly troublesome when aqueous solvents are used as eluents.

Several precautions may aid in controlling this difficulty. The amount of extraneous material eluted varies with the volume of solvent used. Therefore, the eluting solvent should be kept to the minimum required for the complete removal of the components of the sample. The selection of solvent systems which tend to concentrate the fraction to be studied in a narrow band reduces the amount of background material since a smaller area of paper is eluted to obtain the desired fraction.

When it is sufficient to measure the sum of the minor components, the accuracy can be enhanced by using the slit technique and concentrating the less polar materials at the solvent front. Similarly, the more polar components may be concentrated on or near the starting line by the proper choice of solvents.

The limit of sensitivity of the method may be determined by comparing the weight of the sample used and the weight of the background. For example, in ouabain (Sample A), a 10-mg. sample was used and the background weight determined on a blank chromatogram averaged 22 mcg. per cup with extreme variations of ±8 mcg. Since these extremes differ by a maximum of 16 mcg., impurities present in quantities of more than 16 mcg. or 0.16% of a 10-mg. sample and concentrated on a single strip, should produce discernible peaks. The sensitivity is reduced where the impurity is distributed over two or more strips.

If one is interested only in the amount of the main component present, it is not necessary to elute quantitatively the entire chromatogram. The desired result can be obtained by comparing the dry weight of the sample and the dry weight of chief component eluted from the paper.

The elution apparatus described was designed to accommodate an 8 × 8-in. paper cut into 42 strips. Obviously, shorter chromatograms can be used equally well. Long paper may also be used by cutting it into two or more sections and eluting each section separately. In using small samples it may be desirable to use thinner or more narrow paper and with large samples to increase the weight or width of the paper. In the latter case, it will be necessary to increase the height of the elution equipment and the capacity of the cups.

[6] Private communication from L. C. Mitchell of the Food and Drug Administration.

SUMMARY

In a previous publication (1), theory, instrumentation, and procedure were given for the gravimetric determination of the distribution of compounds over an entire paper chromatogram. Some applications of this technique to analysis of steroids are now reported. The method measures components of a drug or other product which are separated by paper chromatography and may be missed by conventional techniques of detection. It eliminates the need for reference standards which for impurities or decomposition products may not be readily available. Precautions in using the method are given. Upon completion of the gravimetric analyses, the isolated fractions were used for additional studies.

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Effects of Some Hypnotic Drugs on Respiration and Oxidative Phosphorylation in Rat Brain

By V. G. ERWIN and HAROLD C. HEIM

Pentobarbital and glutethimide inhibited respiration of rat brain homogenates in the presence of glucose, pyruvate, malate, or glutamate. These effects were not evoked by ethinamate or methyprylon. None of the drugs produced effects on malic dehydrogenase activity, but both pentobarbital and glutethimide inhibited the oxidation of reduced diphosphopyridine nucleotide. Of the four drugs studied only pentobarbital produced significant effects on oxidative phosphorylation by mitochondria.

Although numerous investigators have studied the effects of narcotics on metabolic processes of the brain *in vitro*, the mechanisms by which these drugs evoke pharmacologic effects are not well understood. It has been demonstrated that barbiturates inhibit oxidation of carbohydrate by brain slices and minces (1–4). Other studies have revealed indications that these drugs interfere with hydrogen transport (5, 6) and with the oxidation of pyruvate (7). It has furthermore been shown that barbiturates in concentrations which more closely approach those present in the brain during anesthesia uncouple oxidation from phosphorylation *in vitro* (8).

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TABLE I.—EFFECT OF CERTAIN HYPNOTICS ON RESPIRATION OF HOMOGENATES[a]

Substrate	Drug	Concn.	µl. O_2 Uptake/ 90 min.[b]	Increase (+) or Decrease (−) Due to Drug, %
None	None	...	20 ± 2.2	...
None	Pentobarbital	1×10^{-3} *M*	12 ± 1.4	−40
None	Pentobarbital	5×10^{-4} *M*	16 ± 0.9	...
None	Glutethimide	1×10^{-3} *M*	14 ± 0.6	−30
None	Glutethimide	5×10^{-4} *M*	16 ± 1.2	...
None	Methyprylon	1×10^{-3} *M*	21 ± 1.9	...
None	Ethinamate	1×10^{-3} *M*	19 ± 2.1	...
Glucose	None	...	145 ± 7.2	...
Glucose	Pentobarbital	1×10^{-3} *M*	40 ± 2.5	−72
Glucose	Pentobarbital	5×10^{-4} *M*	77 ± 3.8	−47
Glucose	Glutethimide	1×10^{-3} *M*	85 ± 5.3	−41
Glucose	Glutethimide	5×10^{-4} *M*	118 ± 4.2	−19
Glucose	Methyprylon	1×10^{-3} *M*	142 ± 5.8	...
Glucose	Ethinamate	1×10^{-3} *M*	141 ± 5.3	...
Pyruvate	None	...	79 ± 4.2	...
Pyruvate	Pentobarbital	1×10^{-3} *M*	31 ± 2.0	−60
Pyruvate	Pentobarbital	5×10^{-4} *M*	41 ± 1.4	−48
Pyruvate	Glutethimide	1×10^{-3} *M*	51 ± 3.8	−35
Pyruvate	Glutethimide	5×10^{-4} *M*	73 ± 4.2	...
Pyruvate	Methyprylon	1×10^{-3} *M*	77 ± 4.0	...
Pyruvate	Ethinamate	1×10^{-3} *M*	77 ± 3.3	...
Malate	None	...	30 ± 2.9	...
Malate	Pentobarbital	1×10^{-3} *M*	21 ± 1.6	−30
Malate	Pentobarbital	5×10^{-4} *M*	23 ± 1.0	−23
Malate	Glutethimide	1×10^{-3} *M*	22 ± 2.4	−26
Malate	Glutethimide	5×10^{-4} *M*	23 ± 2.5	−23
Malate	Methyprylon	1×10^{-3} *M*	29 ± 1.6	...
Malate	Ethinamate	1×10^{-3} *M*	28 ± 1.1	...
Glutamate	None	...	33 ± 3.0	...
Glutamate	Pentobarbital	1×10^{-3} *M*	23 ± 1.9	−30
Glutamate	Pentobarbital	5×10^{-4} *M*	28 ± 1.4	...
Glutamate	Glutethimide	1×10^{-3} *M*	26 ± 2.1	−21
Glutamate	Glutethimide	5×10^{-4} *M*	30 ± 2.1	...
Glutamate	Methyprylon	1×10^{-3} *M*	31 ± 1.4	...
Glutamate	Ethinamate	1×10^{-3} *M*	31 ± 2.2	...

[a] Experimental conditions as outlined in the text. [b] Standard deviation. Results significant at the 95% probability level as shown by the Student "t" test.

More recently the hypnotic drugs glutethimide,[1] methyprylon,[2] and ethinamate[3] have been introduced. A search of the literature indicates that the effects of these drugs on metabolic processes of the brain have not been extensively studied. Accordingly, a series of experiments was designed to ascertain whether these drugs evoke effects similar to those of pentobarbital on brain homogenates and mitochondrial preparations.

EXPERIMENTAL

Young, adult Sprague-Dawley rats of both sexes were used as the experimental animals. Each rat was stunned and decapitated, the brain was immediately removed, washed in cold 0.1 *M* phosphate buffer, blotted dry, and weighed on a torsion balance. The brain was then ground in a Ten Broek homogenizer containing sufficient cold 0.1 *M* potassium phosphate buffer, pH 7.4, so that there were 100 mg. of brain per ml. of homogenate.

Mitochondria were isolated from homogenates of whole brain prepared in cold 0.25 *M* sucrose solution according to the method of Brody and Bain (8). Centrifugation was accomplished in a Servall RC-2 centrifuge equipped with an SS-34 rotor. The mitochondrial suspension was adjusted with 0.25 *M* sucrose solution so that each milliliter contained mitochondria representing 200 mg. of brain, wet weight.

Conventional manometric techniques were used for measuring oxygen consumption. In the preparation of the homogenates no more than 10 minutes elapsed between the time the animals were sacrificed and the flasks placed in the bath. All results represent the average of determinations obtained with eight rats and duplicate flasks were used in each instance. The pH of the contents of a representative number of flasks was determined at the end of the experiments to insure that the observed effects were not due to a change in pH.

All flasks used in studying respiration of the homogenates contained in the main compartments 1.0 ml. of 0.1 *M* potassium phosphate buffer (pH 7.4), 0.3 ml. 5×10^{-4} *M* cytochrome c, 0.5 ml. of homogenate, and 0.3 ml. of drug solution to yield the concentrations listed in Table I. Four-tenths of a milliliter of 0.1 *M* substrate was tipped from the sidearm at zero time and the center wells contained 0.2 ml. of 10% KOH together with a strip of filter paper.

When endogenous uptake was determined, water rather than substrate was added from the sidearm.

[1] Marketed as Doriden by Ciba Pharmaceutical Products, Inc.
[2] Marketed as Noludar by Hoffmann LaRoche, Inc.
[3] Marketed as Valmid by Eli Lilly and Co.

TABLE II.—EFFECT OF CERTAIN HYPNOTICS ON OXIDATIVE PHOSPHORYLATION BY RAT BRAIN MITOCHONDRIAL PREPARATIONS WITH PYRUVATE AS SUBSTRATE[a]

	Concn. Drug	μA Oxygen Consumed[b]	μM Phosphate Uptake	P/O Ratio
Controls		1.6 ± 0.3	4.3 ± 0.7	2.7 ± 0.2
Glutethimide	1×10^{-3} M	0.8 ± 0.1	2.1 ± 0.2	2.6 ± 0.3
Methyprylon	1×10^{-3} M	1.6 ± 0.3	4.2 ± 0.3	2.6 ± 0.2
Ethinamate	1×10^{-3} M	1.6 ± 0.2	4.1 ± 0.4	2.5 ± 0.2
Pentobarbital	5×10^{-4} M	1.1 ± 0.2	0.8 ± 0.1	0.7 ± 0.1

[a] Experimental conditions described in text. Each result represents the average obtained with six different animals. [b] Standard deviation; results significant at the 99% probability level as shown by Student "t" test.

With glucose as the substrate the flasks contained the reagents listed above plus 0.1 ml. of 5×10^{-2} M adenosine triphosphate (ATP) and 0.1 ml. of 10^{-2} M $MgCl_2$ in the main compartment, and 0.1 ml. of 10^{-3} M DPN added with the substrate from the sidearm. In the experiments with pyruvate as the substrate flasks contained, in addition to the above reagents, 0.1 ml. of 5×10^{-2} M ATP; 0.1 ml. of 10^{-2} M $MgCl_2$ and 0.1 ml. of 2.5×10^{-2} M nicotinamide all added to the main compartment, while the pyruvate plus 0.1 ml. of 10^{-2} M malate was added from the sidearm.

Water was used to make the final volume of flask contents 3.0 ml. and, with the exception of the experiments with malate which were run at 30°, the temperature of the bath was 37°. The equilibration period was 10 minutes after which the manometers were closed and readings taken at 15-minute intervals for 90 minutes.

Flasks used in the study of oxidative phosphorylation contained, in the main compartment, 0.4 ml. of mitochondrial suspension; 0.5 ml. of 0.1 M phosphate buffer, pH 7.4; 0.1 ml. of 5×10^{-4} M cytochrome c; 0.4 ml. of 0.1 M glycylglycine buffer, pH 7.4; 0.1 ml. of 1.5×10^{-2} M DPN; 0.1 ml. of 0.5 M KCl; 0.1 ml. of 0.3 M $MgCl_2$; 0.1 ml. of 0.8 M glucose; 0.1 ml. of hexokinase solution containing 600 units per ml.; 0.1 ml. of 5×10^{-2} M ATP; and 0.1 ml. of 0.5 M NaF. Three-tenths of a milliliter of 0.1 M pyruvate together with 0.1 ml. of 10^{-2} M malate was added as substrate. Three-tenths of a milliliter of drug solution was added to yield the concentrations listed in Table II. The center wells contained 0.2 ml. of 10% KOH together with a strip of filter paper, and the temperature of the bath was 30°.

The control flasks, which contained water in place of drug solution, were removed at the end of the 10-minute equilibration period and immediately placed in ice, following which 0.5 ml. of chilled 20% trichloracetic acid (TCA) was added to denature proteins. The same procedure was followed with the flasks containing the drugs after the oxygen uptake had been measured for 30 minutes. The contents of the TCA-treated flasks were pipetted into centrifuge tubes and the denatured protein separated by centrifugation at 3000 × g for 10 minutes. Inorganic phosphate was determined in the supernatant fluid by the method of Fiske and SubbaRow (9). A Beckman model DU spectrophotometer was used and the absorbance was determined at 660 mμ. The inorganic phosphate uptake was determined as the difference between the amount of phosphate in the vessels to which TCA had been added at the end of the equilibration period and those to which TCA was added after measuring oxygen uptake for 30 minutes. The phosphate-to-oxygen ratio was calculated as the ratio of micromoles of inorganic phosphate uptake to the microatoms of oxygen consumed, in conformance to the methods followed by Brody and Bain (8).

The effect of the drugs on malic dehydrogenase activity was determined by the method of Ochoa (10). The oxidation of dihydro-β-diphosphopyridine nucleotide (DPNH) to diphosphopyridine nucleotide (DPN) with the concomitant reduction of oxaloacetate to malate was measured, in the presence and in the absence of the drugs, by following the decrease in absorbance at 340 mμ with the Beckman model DU spectrophotometer. In these experiments the reaction mixture contained, in a total volume of 3.0 ml., 40 units of malic dehydrogenase, 5×10^{-5} M DPNH, 5×10^{-5} M oxaloacetate, and 10^{-3} M of the various drugs.

In those instances where the drugs produced an inhibitory effect on the respiration of homogenates, additional experiments were performed to determine

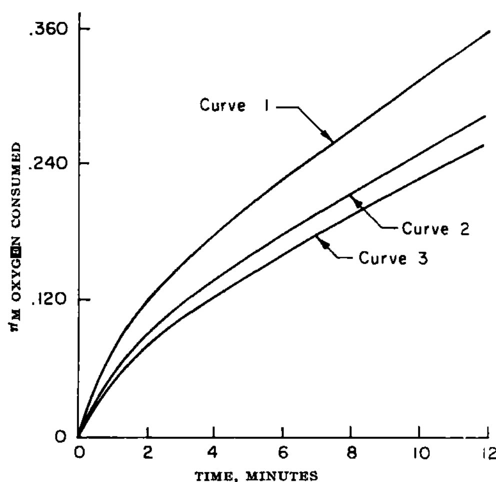

Fig. 1.—The effect of pentobarbital and glutethimide on respiration of brain mitochondria in the presence of malate. Cell of the polarograph contained 0.5 ml. of 0.01 M phosphate buffer; 0.1 ml. of 5×10^{-4} M cytochrome c; 0.1 ml. of 0.05 M ATP; 0.1 ml. of 0.5 M KCl; 0.1 ml. of 1.5×10^{-2} M DPN; 0.1 ml. of 10^{-2} M $MgCl_2$; 0.3 ml. of 0.1 M malate; and 0.2 ml. mitochondrial suspension representing 100 mg. brain. Drug, when present, added as 0.2 ml. of 10^{-2} M. Water added to make volume 2.0 ml. Temperature 25°. Results represent averages obtained with six animals. Key: curve 1, control (no drug added); curve 2, glutethimide; and curve 3, pentobarbital.

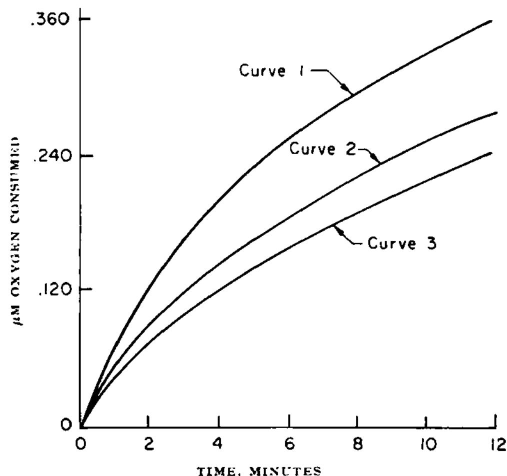

Fig. 2.—The effect of pentobarbital and glutethimide on respiration of brain mitochondria in the presence of pyruvate. Cell contents same as outlined in Fig. 1 with 0.3 ml. of 0.1 *M* pyruvate added. Experimental conditions identical to those used for Fig. 1. Results represent averages obtained with six animals. Key: curve 1, control (no drug added); curve 2, glutethimide; and curve 3, pentobarbital.

the effects of the rate of oxygen uptake by mitochondria. This was carried out by measuring the oxygen uptake polarographically, using a vibrating platinum electrode instrument[4] adapted from the method outlined by Chance (11). With such an instrument it was possible to measure the consumption of extremely small amounts of oxygen accurately over very short time intervals. The effects of pentobarbital and glutethimide on the respiration of mitochondria in the presence of malate, pyruvate, or DPNH as substrates were thus determined.

RESULTS

The data included in Table I indicate that either pentobarbital or glutethimide, at concentrations of 10^{-3} *M* and 5×10^{-4} *M*, inhibit the endogenous oxygen uptake of homogenates as well as oxygen uptake in the presence of added glucose, pyruvate, malate, or glutamate. Neither ethinamate nor methyprylon (at these concentrations) evoked appreciable effect. As further shown by Table I, pentobarbital produced somewhat more marked effects on respiration than did glutethimide.

Figures 1–3 illustrate the effects of pentobarbital and glutethimide on the oxidation of malate, pyruvate, and DPNH by brain mitochondrial preparations. It is apparent that both glutethimide and pentobarbital significantly depress the rate of oxidation of these substrates.

Table II shows that, of the four drugs studied, only pentobarbital evoked significant effects on oxidative phosphorylation by brain mitochondria.

None of the drugs produced significant effects on the activity of a purified malic dehydrogenase obtained from commercial sources.[5]

[4] Model K Oxygraph, Gilson Medical Electronics Co., Madison, Wis.
[5] Sigma Chemical Co., St. Louis, Mo.

DISCUSSION

The oxidation of malate and glutamate by homogenates was inhibited by pentobarbital or glutethimide. Other investigators (7) have shown that pentobarbital does not block the oxidation of glucose to pyruvate. It has also been demonstrated that the major pathway for the metabolism of glucose by the brain occurs *via* glycolysis to pyruvate (12) with subsequent oxidation by way of the tricarboxylic acid cycle. It might be expected, therefore, that the inhibitory effect evoked on the oxidation of glucose would be very similar in magnitude to the effect on the oxidation of pyruvate. In this study it was found that pentobarbital inhibited the oxidation of glucose to about the same degree as was observed with pyruvate.

Although the oxidation of the substrates included in this study is dependent upon specific dehydrogenase systems, it is not correct to deduce that the inhibitory effects produced by the drugs are due to interference at the dehydrogenase level because the observations made with homogenates are not necessarily the same as those made with purified enzyme systems. The use of more discrete preparations, such as mitochondria, affords a means of measuring oxidation free from the influence of many of the reactions which simultaneously occur in homogenates. Polarographic determination of oxygen consumption by mitochondria showed that pentobarbital and glutethimide were inhibitory in the presence of added malate, pyruvate, or DPNH, while the activity of a purified malic dehydrogenase preparation was not altered. Such observations

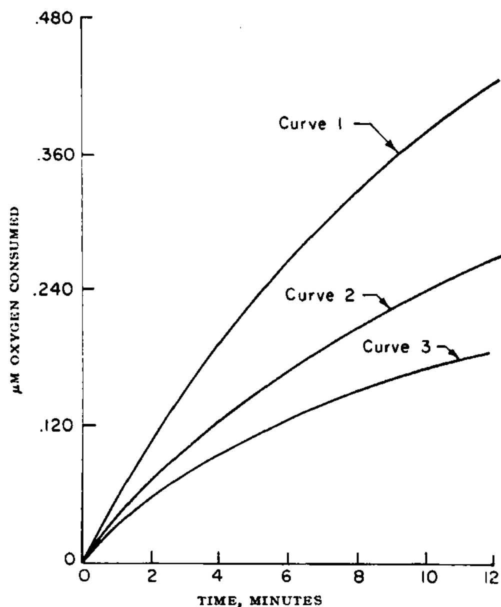

Fig. 3.—The effect of pentobarbital and glutethimide on the oxidation of reduced diphosphopyridine nucleotide (DPNH). Cell contents same as those for Fig. 1 except that the only substrate added was 0.3 ml. of 10^{-2} *M* DPNH. Experimental conditions identical to those used for Fig. 1. Results represent averages obtained with six animals. Key: curve 1, control (no drug added); curve 2, glutethimide; and curve 3, pentobarbital.

indicate that dehydrogenases are not directly inhibited by these drugs and lead to a consideration of possible effects of the drugs at the level of hydrogen transport.

It has been well established that mitochondria are rich in the enzymes of the tricarboxylic acid cycle, the electron transport and accompanying phosphorylating systems, and the cofactors associated with these enzymes (13, 14). The malate and pyruvic dehydrogenases both require DPN as a cofactor and utilize the same cytochrome c reductase system as the primary pathway for electron transport. Thus, the main difference between the oxidation of pyruvate and malate involves the dehydrogenases which transfer protons and electrons from these substrates to DPN. Pentobarbital or glutethimide, then, may conceivably produce an inhibition of oxygen uptake in the presence of malate or pyruvate by blocking at the dehydrogenase level or at some point in the hydrogen transport system beyond the step responsible for the reduction of DPN. The data in Fig. 3 reveal that the rate of oxygen uptake in the presence of added DPNH was inhibited by both pentobarbital and glutethimide.

The succinic oxidase system, in the presence of cytochrome c, will oxidize *p*-phenylene diamine with electrons being transferred directly to the cytochrome c, thus involving a bypass of the succinate-cytochrome b complex (15). It has been demonstrated that glutethimide (16) or pentobarbital (17) do not inhibit the oxidation of succinate or of *p*-phenylene diamine. It may be inferred, therefore, that the dehydrogenases which act on *p*-phenylene diamine or succinate transfer electrons to a point beyond the DPNH-dehydrogenase step in the electron transport system. There is evidence (18) that pentobarbital does not affect the DPN-linked dehydrogenases of the tricarboxylic acid cycle. The results of this study indicate that neither pentobarbital nor glutethimide inhibit the activity of a purified malic dehydrogenase system. However, the drugs inhibit the oxidation of DPNH and of other substrates which require DPN-linked dehydrogenases. Therefore, the results reported herein suggest that the effects produced by either pentobarbital or glutethimide *in vitro* are elicited at the level of the electron transport system between DPN and cytochrome c.

Of the four drugs studied, only pentobarbital was found to uncouple oxidation from phosphorylation. The concept of such uncoupling seems to be more widely accepted as a possible explanation of central nervous system depression than is the inhibitory effect of this drug on respiratory enzymes, perhaps because concentrations required to impair oxidative phosphorylation *in vitro* more closely approach the concentrations which produce *in vivo* effects. It is pertinent to point out, however, that not all depressants of the central nervous system are uncoupling agents, as shown by the fact that neither chloral hydrate nor paraldehyde uncouple oxidation from phosphorylation (19).

It seems of interest to note that neither ethinamate nor methyprylon had an effect on the oxidative processes included in this study even though the hypnotic potency of these drugs has been reported to be comparable to that of glutethimide (20–22).

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