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INHIBITION OF STIMULATED CEREBRAL CORTEX RESPIRATION AND GLYCOLYSIS BY CHOLINOLYTIC DRUGS*

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Abstract—The role of central cholinergic synapses in excitable tissue is suggested from the results described. Six cholinolytic drugs, including Ditran (JB-329), are shown to block stimulated respiration of rat and guinea pig cerebral cortex (at concentrations 5×10^{-4} to 10^{-5} M) without demonstrable effect on unstimulated tissue. One of these drugs (JB-840) was studied for effects on electrically stimulated (10 to 12 V, 120 pulses/sec) and potassium ion (105 mM) stimulated glycolysis and respiration which are selectively depressed at similar concentrations. In addition a simple device for sectioning brain tissue is described.

IT IS generally accepted that respiration and glycolysis supply most of the energy requirements of brain[1] and that glucose is the preferred substrate. Although other substrates can be utilized, respiration is markedly less stable under these conditions.[2] Despite this marked glucose dependency, levels of oxygen and glucose consumption *in vitro* are only half those predicted from *in vivo* estimates.[3] In the presence of high potassium (105 mM) ion concentration[4] or as a result of applying electrical impulses[5] an additional quantity of oxygen is taken up by respiring cerebral cortex slices, such that the rate of consumption more nearly approximates the value *in vivo*.[6, 7] This is paralleled by a proportionate increase in glucose consumed and lactic acid produced. If brain tissues are maintained at ice temperature for varying periods in the absence of substrate and adequate oxygenation, responsiveness to stimulus is lost,[8] although unstimulated levels of respiration and glycolysis appear normal with added substrate. These observations suggest that lower metabolic levels of activity are required for "metabolic maintenance" and that a higher level, associated with "active processes"—e.g. tissue excitability, ion transport, central synaptic transmission, etc.—is associated with cellular integrity. Drugs having specific actions on functions ascribable to the central nervous system may selectively interfere with active processes without seeming to disturb metabolic maintenance levels of biochemical activity.

Much attention has recently been devoted to the actions of "psychotropic" drugs, and the numerous reviews and symposia[9–11] reflect an intensive effort to find satisfactory explanations for their diverse actions at the biochemical level. This paper describes the selective inhibition of stimulated aerobic glycolysis and respiration by

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six cholinolytic drugs,[12] some of which [JB-318; JB-329 (Ditran)] produce marked psychotic-like disturbances in otherwise normal human subjects.[13]

EXPERIMENTAL

Compounds studied

The following cholinolytic compounds (Fig. 1) were the generous gift of Dr. John Biel of the Lakeside Laboratories, Madison, Wis.: JB-840 (N-methyl-3-piperidyl-phenyl*cyclo*hexyl glycolate hydrochloride); JB-336 (N-methyl 3-piperidylbenzilate); JB-344 (N-methyl-3-piperidylphenyl-2-thienyl glycolate hydrochloride); JB-329 (Ditran) (N-ethyl-3-piperidylphenyl*cyclo*pentyl glycolate hydrochloride); JB-318 (N-ethyl-3-piperidylbenzilate hydrochloride). Dr. Y. Pan, Chas. Pfizer, Groton, Conn., supplied a 1-g sample of Daricon (1-methyl-1,4,5,6-tetrahydro-2-pyrimidylmethyl *cyclo*hexylphenylglycolate hydrochloride). Small (ca. 10-mg) samples were weighed and diluted with cold buffer just prior to use.

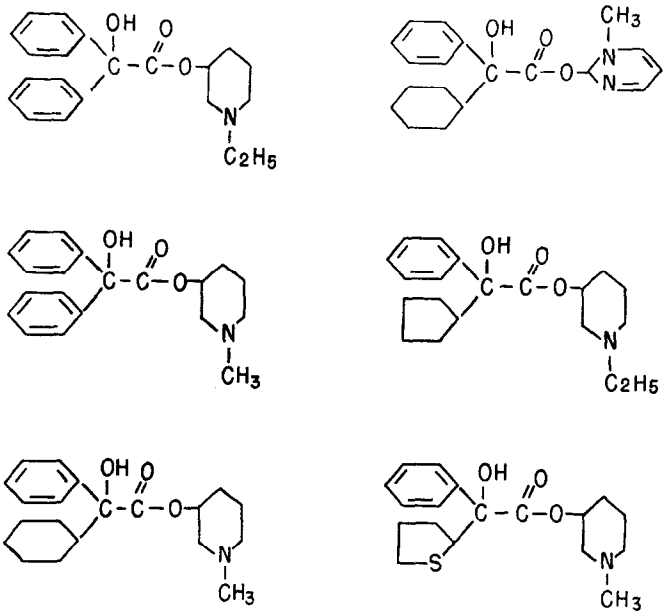

FIG. 1. Structural similarities of the six anticholinergic drugs compared. For a chemical description see text.

Incubation medium. The mixture contained Tris buffer (0·038 M), pH 7·4, sodium chloride (0·127 M), potassium chloride (0·005 M), magnesium sulfate (0·0013 M), calcium chloride (0·0027 M), and disodium phosphate (0·0031 M). Glucose (0·010 M) was added to the cold mixture, and the solution was then gassed for 10 min with oxygen. All studies were carried out in a 100 % oxygen atmosphere.

Potassium stimulation. To compare the effect of various drugs on stimulated respiration, aqueous solutions of the drug were added to the tissue medium prior to incubation and, after a control period of respiration, 0·2 ml KCl (2·63 M) was added from a side arm (final volume 5·0 ml, 105 mM). In subsequent comparative studies

with compound JB-840, drug and KCl solutions were added initially to the main vessel compartment prior to introducing the tissue. This simplified subsequent rate calculations; all analytical results are calculated from the time the vessels enter the bath until removal for sampling. In electrical stimulation the drug was added to the tissue but current was not applied until after gassing. In comparing the effect of various drugs (Table 1) on stimulation, a control period of respiration (40 min) was followed by a longer (1-hr) period of stimulation. In the comparative studies (Table 3) stimulus was applied immediately after gassing and allowance made for glycolytic changes occurring before that time.

Glucose was determined on the flask contents at the end of the incubation period by the Nelson modification[14] of the Somogyi method. To obtain consistent results it was found necessary to rinse the tissue slices and holders three times with glucose-free salines prior to placing them in the flasks. The drugs studied did not interfere in any of the analyses, although controls were routinely run containing the highest drug concentration present.

Lactic acid was assayed by the method of Barker and Summerson.[15] Rapid cooling of the sample tubes in dry ice–acetone prevented spattering and speeded the addition of sulfuric acid.

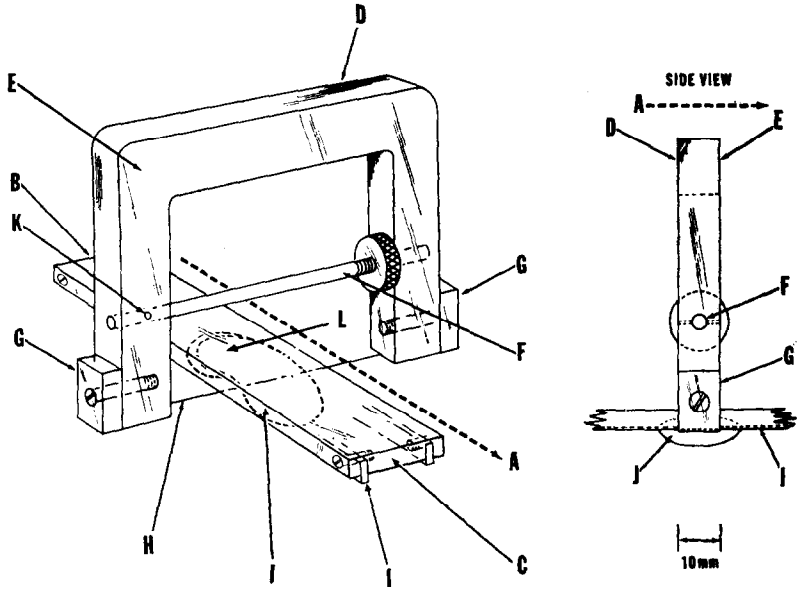

FIG. 2. A simplified brain-tissue slicer. For details of its components and manipulation see text.

Tissue slice technique. Unlike liver and kidney, slicing brain tissue with conventional apparatus (e.g. Stadie–Riggs) proved less than satisfactory. Brain slicing was therefore performed with the apparatus* shown in Fig. 2, drawn approximately to scale. It consists of a cutting frame 62 × 50 × 6 mm and a cutting block 31 × 75 × 6

* Designed in collaboration with Dr. E. J. Schantz, Allied Sciences Div., USABRDL, Ft. Detrick, Md.

mm fashioned from 1/4-in. clear plastic (Lucite). The cutting block has two parallel aluminium alloy strips (I) 20 mm apart, extending the length of the block and protruding 0·30 mm from the Lucite surface, which serve as guides for the cutting wire and assist in controlling thickness of slice. Smaller (23 × 75 × 6 mm) cutting blocks for rat brain have also been made, but the larger blocks are satisfactory for most purposes. The cutting frame is designed so that the proper wire tension essential for reproducible slice thickness is readily attained. Holes 1/8 in. in diameter are drilled through each arm of the frame, approximately 3/4 in. from the end. The hole on the right is then reamed out approximately 1/64 in. oversize. An aluminum alloy rod (F) 1/8 in. in diameter and approximately as long as the frame width, is threaded for a third of its length and inserted through the two holes. A knurled nut is threaded on and, after the rod is centered laterally in the frame, it is anchored (K) with a stainless steel set screw. To install the 36-gauge (S.W.G.) silver cutting wire the knurled nut is lightly pressed against the frame, and one end of the wire from a spool is fastened to the side of the frame with a Lucite clamp (G) and tightened with a stainless steel machine screw (No. 256). The wire under manual tension is then attached to the other arm in a similar manner and cut free from the spool; final adjustment of cutting wire tension is made by rotating the knurled nut.

Male Wistar rats, weighing 200 to 250 g, or male guinea pigs (300 to 400 g) were killed by decapitation and their heads plunged into chopped ice. After rapid removal from the brain case the brains were freed from blood vessels and dura and dropped into cold Tris–Ringer–glucose saline (see Incubation Medium), divided into two cerebral hemispheres, and the brain stem removed with small curved forceps.

The cutting operation was performed in a Petri dish (140 mm size) containing Tris–Ringer–glucose solution. The cerebral hemisphere (J), free from brain stem, rested upon an 11·0-cm circle of Whatman No. 1 filter paper. The cutting block was placed over the tissue and held by the middle finger and thumb at (B) and (C) respectively. The slicer frame was grasped by the other index finger (D) and thumb (E). The hemisphere was gently compressed by pressure applied to the block, and the wire was carefully drawn through the tissue in the direction shown (A), so that a slice (L) was formed from the portion of brain tissue that extended into the recess between the aluminum strips. The newly cut slice was removed with a blunt forceps and placed in a Petri dish containing ice-cold Tris–Ringer–glucose solution. This process can be repeated to yield as many as four thin slices [0·35 ± 0·05 mm (avg. dev.)] from a single hemisphere of brain; routinely the three outer slices were used. Slice thickness was determined by the method of Field.[16]

Tissue dry weights were estimated from "apparent" wet weight measurements. A trimmed tissue slice, freed from loosely adhering fluid by passing it three to four times over a clean, dry, glass surface, was suspended from a wire loop and weighed ("apparent" wet weight) on a Roller–Smith microtissue balance. Imbibition of water can result in wet weights approximately 22 per cent higher than those of tissue samples taken directly from fresh brain. Many (56) varied sizes of cortex slice were dried at 105° to constant weights, and the values were plotted versus apparent wet weights. The slope of the line of best fit was estimated to be 0·147 ± 0·014 (S.D.) for a rat and guinea pig brain. This compares favorably with the conversion factor estimated by McIlwain and Rodnight,[17] and it was therefore used in all wet weight:dry weight estimations.

Apparatus

McIlwain flasks[18] with standard taper (17/26) joints and fitted with side arms containing platinum–tungsten stub electrodes were fabricated by A. W. Dixon Co., 20 Annerley Station, London S.E.20, and the small grid electrodes[19] were obtained from H. L. Buddle, 46 Cedarcroft Road, Chessington, Surrey, England. The vessels were calibrated with mercury in the usual way and the manometer volumes determined with a Lazarow manometer calibrator, Micro Instrument Company, Cleveland, Ohio. Grid-electrode volumes were estimated from Archimedes' principle:

$$\frac{\text{weight in air} - \text{weight in water}}{\text{density of } H_2O} = \text{volume of electrode}$$

Combined flask and manometer volumes were corrected for this volume change in calculating vessel constants.

Initially, electrical contacts from the stimulator to the grid electrodes were made through columns of mercury in the flask electrode side arms; later, copper leads were soldered directly to bright tungsten surfaces (important for good electrical contact). Removal of oxide from the tungsten was accomplished by placing a small (2 ml) volume of concentrated sodium hydroxide (10 M) in contact with the tungsten, running leads from an a.c. variable transformer to a copper wire immersed in the alkali and to the platinum wire inside the flask. Current at 2 to 4 V was passed for 2 to 3 min through the solution and a metallic-gray surface free from oxide was obtained. A fluxless solder joined the copper wires to the tungsten stubs which gave reasonably good results; a thermo-setting resin containing a high (99%) silver content was tried, but the results were variable. The poor wetting property of tungsten made it difficult to obtain connections which did not develop high resistance.* As a precaution, electrical resistance of each vessel lead was checked with a d.c. ohmmeter before and after an experiment, and only results from flasks that showed negligible resistance change are reported. Resistance during an experiment may also be measured with a precalibrated current probe (Tektronix, P6016).

Gold plating of platinum. The low hydrogen overvoltage of platinum which affects current efficiency[20, 21] makes gold plating desirable,[22] and plating platinum with 24-carat is achieved within a matter of minutes. Flasks are filled with a solution of gold chloride (900 mg), dissolved in 50 ml distilled water containing potassium cyanide (3·3 g), or with a gold-plating solution obtained commercially (L. D. Caulke Co., Milford, Del.). The flask and its contents are warmed to 70° on a steam bath, and the bright surface of a 10 cm × 0·5 cm zinc rod is touched to the platinum wire. After decanting the solution the resultant dull gold plate is buffed with moistened sodium bicarbonate on absorbent cotton and, after thorough rinsing, the flasks can be used several times before replating.

Tissue stimulator. Early results with a Grass model S-4 stimulator with square wave pulses (100 pulses/sec, reversing polarity) at 2 to 3 V potential were extremely variable, and stimulation could not be sustained over a reasonable period. The stimulator apparently lacks sufficient power or has too high an internal impedance for the

* A paper in preparation describes a new vessel that eliminates many of the difficulties inherent in the present design.

low tissue impedances encountered. Best results were obtained from two single-channel integrated square-wave pulse generators built by W. Manchester (USACRDL, ACC, Md.) from a preprint of a paper kindly sent by McIlwain.[23] The parts included Sigma type relays GF X 2A2A-5000-G-Sil and 72AO-400TG-TCP. Pulses (120 pulses/sec, 0 to 24 V potential) of variable duration (0 to 0·75 msec) were obtained; routinely a pulse duration of 0·4 msec and 10 to 12 V potential was employed.

RESULTS AND DISCUSSION

Methods that produce metabolic increases in cerebral cortex slices appear especially applicable to the study of drugs acting on the central nervous systems.[24, 25] Dickens and Greville[26] and Ashford and Dixon[27] observed that high potassium ion concentrations markedly stimulate brain tissue respiration. Similar response to electrical stimulation has been utilized by McIlwain and Greengard in studies of mescaline and lysergic acid diethylamide (LSD-25) and other centrally-acting drugs.[24] Wollenberger demonstrated that protoveratrine also stimulated respiration[28] and concluded that it involved potassium ions. Preliminary studies in this laboratory demonstrated that protoveratrine (2×10^{-6} M) produced a small (38%) increase in oxygen consumption in rat brain slices which was sensitive to cholinolytic drugs, but since this stimulation is concentration dependent (6×10^{-6} M protoveratrine-A inhibits respiration) and depends on potassium ions, it was not investigated further.

Biochemical studies of drug action frequently assume that a specific enzyme or system is to be interfered with and that it should be possible, therefore, to demonstrate such interference *in vitro*. It is equally acceptable that the primary action may be on a process requiring an intact structure and that biochemical changes are secondary in nature, reflecting interference with the primary mechanism. In this regard we may visualize acetylcholine accumulation as the result of the inactivation of cholinesterase and, conversely, the antagonism of curare action on neuromuscular activity by physostigmine.

It may be seen from results obtained with rat brain (Table 1) that respiratory activity *in vitro* in Tris buffer can be increased by high potassium ion concentration, confirming the observation of Elliott[3] that respiratory rates *in vitro* are considerably lower than values *in vivo*, suggesting the loss of some component in the system. All the drugs studied depressed stimulated respiration; the most potent appeared to be Ditran (JB-329), JB-840, and JB-318; JB-344 had the smallest (26%) effect. Especially noteworthy are the apparent selective effects on respiration, since there was no demonstrable change in unstimulated respiration (Tables 1–3) with any drug studied. This perhaps explains the lack of drug effects reported for certain members of this class by Abood *et al.*[29] McIlwain and Greengard[24] and Quastel[25] differ concerning similarities between electrical and potassium ion stimulation. Results obtained in Table 2 show electrical stimulation to be somewhat more sensitive to the action of these drugs since there is greater depression of extra respiration at all concentrations studied. These results contrast with McIlwain's earlier findings[30] in which he failed to demonstrate an effect of atropine (L-hyoscyamine) and scopolamine (L-hyoscine) on potassium-stimulated respiration. It has recently been shown that potassium produces a spreading depolarization,[31] whereas electrical effects are more discrete, and this perhaps accounts for the quantitative differences reported.

Energy in nervous tissue appears to be derived principally from aerobic glycolysis.[32] Species differences which might be inferred from the use of rat (Table 1) and guinea pig (Table 2) are ruled out in later studies. A comparison is made (Table 3) of the two forms of stimulation which show that increases in respiration are paralleled by changes in glucose consumption and lactic acid production. The marked depression of stimulated respiration by JB-840, noted earlier, is accompanied by a significant ($P < 0.001$) decrease in glucose utilized and lactate produced. There are no significant effects

TABLE 1. INHIBITORY EFFECT OF CHOLINOLYTICS ON POTASSIUM ION-STIMULATED CEREBRAL CORTEX RESPIRATION*

Drug	Conc. (moles/L)	Potassium ion (105 mM) stimulation Unstimulated†	Stimulated†	Inhibition‡ (%)
Control		495	830	
JB-840	5×10^{-4}	465	593	62
	1×10^{-4}	495	920	0
Control		410	800	
JB-336	5×10^{-4}	400	620	44
	1×10^{-4}	400	738	13
	5×10^{-5}	400	700	23
Control		410	800	
JB-318	5×10^{-4}	385	515	64
	$2{\cdot}5 \times 10^{-4}$	396	640	37
	1×10^{-4}	366	685	18
	5×10^{-5}	390	780	0
Control		495	830	
JB-329	5×10^{-4}	490	620	61
	1×10^{-4}	424	745	4
Control		410	800	
JB-344	5×10^{-4}	390	680	26
	$2{\cdot}5 \times 10^{-4}$	390	800	0
	1×10^{-4}	364	700	14
Control		370	675	
		520	830	
Daricon	5×10^{-4}	526	675	52
	1×10^{-4}	365	580	31
	1×10^{-5}	380	645	15

* Rat brain.

† Rates are expressed as μmoles O_2/g/hr where g = grams dry weight = approx. wet wt $\times$ C_f(dry wt/wet wt = 0·147).

‡ $$\text{Inhibition (\%)} = \frac{\text{control (stim. } - \text{ unstim.)} - \text{drug (stim. } - \text{ unstim.)}}{\text{control (stim. } - \text{ unstim.)}} \times 100.$$

($P = 0.05$) on these parameters in unstimulated tissue. As noted before, there are quantitative differences of drug effect on the two forms of stimulation, but it is not certain whether the recovery of respiration before glycolysis is statistically significant ($P \equiv 0.05$) in potassium stimulation. It does appear, however, that the elevated glycolytic levels caused by electrical stimulation are more drug sensitive.

It was reported in preliminary studies[12] that the omission of calcium from the incubation medium caused an increase in respiration which was not depressed by JB-840. Smith and Abood have reported that the appearance of lactic acid from intact muscle bathed in a calcium-deficient medium is depressed by an analog of Ditran, PMCG.[33] As total lactates were not reported, this may have been a nonspecific effect on membrane permeability which is markedly influenced by calcium ion concentration.[34] It is, however, of interest that Adams and Quastel[35] demonstrated that a variety of organic bases could replace calcium ions in supporting anaerobic glycolysis, and their relative potencies were a function of pK. Anaerobic glycolysis is different from aerobic glycolysis in cerebral tissues in that electrical pulses, excess potassium or ammonium ions, the presence of glutamate, or the absence of calcium all decrease anaerobic glycolysis; aerobically they cause its increase.[2a] The depression of respiration and aerobic glycolysis seen with these drugs may cause stimulation of anaerobic counterparts.

TABLE 2. INHIBITORY EFFECT OF CHOLINOLYTICS ON ELECTRICALLY STIMULATED CEREBRAL CORTEX RESPIRATION*

Drug	Conc. (moles/L)	Electrical stimulation Unstimulated†	Stimulated†	Inhibition (%)
Control		322	595	
JB-840	5×10^{-4}	360	397	87
	5×10^{-5}	362	483	56
Control		405	720	
JB-336	5×10^{-4}	340	455	64
	1×10^{-4}	350	505	51
Control		405	720	
JB-318	5×10^{-4}	340	390	84
	1×10^{-4}	375	485	65
Control		313	500	
JB-329	5×10^{-4}	330	330	100
	1×10^{-4}	300	420	36
Control		315	530	
JB-344	5×10^{-4}	335	430	56
	1×10^{-4}	310	415	51
Control		315	530	
Daricon	5×10^{-4}	375	440	70
	1×10^{-4}	325	455	40

* Guinea pig brain.

† Rates are expressed as μmoles O_2/g/hr.

An excellent review concerning benzilate and substituted phenylglycollate esters has recently appeared[11] which covers most of what is known about these compounds, and Hunter and Lowry[36] have published an excellent discussion of alternative drug action in the central nervous system. Garrattini *et al.* have shown[37] that benzilic acid ethers inhibit cholinacetylase *in vitro*, and Wagner-Jauregg demonstrated that certain atropine-like spasmolytics interfere with acetyl-coenzyme A-dependent systems[38]

TABLE 3. INFLUENCE OF JB-840 ON CEREBRAL CORTEX RESPIRATION AND GLYCOLYSIS*

Drug conc.	Unstimulated			K^+ ion (105 mM) stimulation			Electrical (12 V) stimulation		
	Oxygen uptake	Lactate formed	Glucose utilized	Oxygen uptake	Lactate formed	Glucose utilized	Oxygen uptake	Lactate formed	Glucose utilized
Control	345 ± 34	183 ± 37	147 ± 17	704 ± 35	455 ± 30	307 ± 22	664 ± 72	341 ± 38	298 ± 52
5×10^{-4} M	378 ± 40	193 ± 64	153 ± 15	398 ± 40	230 ± 50	181 ± 41	379 ± 72	258 ± 52	154 ± 51
10^{-4} M	315	202 ± 29	162	586 ± 46	309 ± 30	169 ± 40	467 ± 95	225 ± 37	150 ± 55
10^{-5} M	385		168	683 ± 8	452 ± 3	291 ± 40	630 ± 68	277 ± 38	198 ± 40
10^{-6} M	330	201	160	613 ± 24	468 ± 6	277 ± 43	627 ± 65	295 ± 37	213 ± 33
10^{-7} M	328		148	675 ± 42	481 ± 2	291 ± 42	656 ± 90	348 ± 29	271 ± 48
	339 ± 35	199 ± 43	158 ± 18						

* Rates expressed as μmoles/dry g/hr, ± mean and standard deviations; male guinea-pig cortex. Substrate: glucose ($5{\cdot}5 \times 10^{-3}$ M)

which may be relevant since Kini and Quastel[39] have proposed that potassium stimulates processes associated with pyruvate utilization via the Krebs cycle. It is possible that drug-sensitive enzymes in major or alternative metabolic pathways go undetected, unless a shift in steady-state levels caused by stimulation makes them rate limiting. In this situation, inhibition would lower metabolic acitivity to the unstimulated condition and would explain why there is no depression of unstimulated tissue metabolism when these enzymes are no longer rate limiting.

Cholinolytic agents act on ganglia and peripherally on smooth muscle by blocking the actions of acetylcholine.[40] There is considerable disagreement as to the role of acetylcholine in the central nervous system, but there is a growing body of evidence that central cholinergic neurons do exist[40]; and that cholinergic[41] and anticholinergic substances produce marked EEG[42] and mood[43] changes. Feldberg has reviewed the evidence for "bound" and "free" acetylcholine, and Whittaker[44] and DeRobertis[45] have presented convincing anatomical evidence of this. Quastel[25] and McIlwain and Greengard[24] have shown that stimulation of cerebral slices leads to a release of bound acetylcholine and an increase in the free form. It is tempting to speculate that Ditran and other cholinolytic agents act upon this mechanism. It may be that the arrival *in situ* of nerve impulses from lower centers triggers the release of acetylcholine, producing depolarization of cholinergic central synapses concomitant with egress of potassium and influx of sodium ions. One of us (J.T.C.) in collaboration with McIlwain has demonstrated significant changes in potassium flux with electrical stimulation.[46] During the repolarization phase that follows the passage of an impulse, added energy is required to re-establish ionic gradients, and this need is reflected in increased respiration and glycolysis. Blocking the primary electrical events negates this secondary effect and only "maintenance" levels of metabolic activity are observed. It is reasonable to suspect such a cholinergic mechanism since Gershon[47] has demonstrated clinically the reversal of psychic effects produced by Ditran with the potent anticholinesterase 1,2,3,4-tetrahydro-5-amino acridine,[48] which in laboratory animals can antagonize the pharmacologic actions of atropine and morphine. Its action might be similar to the action that physostigmine produces peripherally against curare action. It is recognized that much work needs to be done before such speculations can be secured by experimental fact. The concentrations of drug employed in this study are probably much higher than needed to produce the subtle derangements associated with disturbances in the conscious state. It is doubtful that significant changes in respiration and glycolysis can be demonstrated *in vivo* with these drugs, especially when one considers the great number of cells participating and the gross heterogeneity of functional and nonfunctional structures that contribute to over-all metabolic activity of our sensorium.

In summary it can be said that cholinolytic drugs such as Ditran, known to cause mental disturbance in human subjects, are without apparent effect on unstimulated cerebral cortex respiration and aerobic glycolysis *in vitro*.

In rat and guinea pig cerebral cortex, potassium ions and electrical stimulation cause marked increases in respiration and glucose consumption and a proportionate rise in lactic acid production. Ditran, JB-840, and four other cholinolytic agents depress stimulated respiration to varying degrees depending upon the drug and nature of stimulus.

A tentative explanation is either a blockade of cholinergic central synapses or inhibition of drug-sensitive enzyme which is only apparent when a change in steady state makes this system rate limiting—i.e. during stimulation.

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