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THE EFFECT OF CHLOROBUTANOL ON THE RESPIRATORY METABOLISM AND ON THE NORMAL PROPERTIES OF ISOLATED MITOCHONDRIA*

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Abstract—The possible site of action of chlorobutanol upon the respiratory metabolism was investigated in rat heart, cerebrum, and cerebellum preparations. The behavior of this compound on respiration of slices, homogenates, and mitochondria showed that its inhibitory effect was more evident when NAD-dependent dehydrogenases were involved in the primary oxidation of substrates, although a more drastic effect of chlorobutanol on the mitochondria could be obtained, since chlorobutanol is also an antiseptic. Nevertheless, chlorobutanol in suitable amounts was used to determine its effect on the normal properties of mitochondria—respiration, oxidative phosphorylation, respiratory control coefficient—and on the steady-state levels of cytochrome b from heart sarcosomes.

CHLOROBUTANOL or chloretone (1,1,1-trichloro-2-methyl-2-propanol) has been used for many years as a preservative in solutions of biological drugs, and for tissue extracts. It possesses a strong antiseptic action for which it is more widely used and known, and it has been used orally or intravenously as an anesthetic for laboratory animals. It is also a fairly effective substance as a preservative in organic preparations, and has a paralyzing effect on the heart muscle.[1]

The mechanism of action of chlorobutanol has been the subject of several papers. Quastel and Wheatley[2] found that narcotics, including chlorobutanol, inhibit the oxidation by brain of glucose, lactate, pyruvate and, to a certain extent, glutamic acid, but not of succinate. They also obtained[3] a 70-per cent reversibility of the strong inhibitory actions of the narcotics chloretone, luminal, and hyoscine on brain respiration by washing pretreated brain slices in a phosphate–glucose–saline medium.

Jowett and Quastel,[4] on the other hand showed that luminal, chloretone, and Evipal inhibited the respiration of liver, kidney, and diaphragm both in the presence and absence of substrate. The stage of oxidation appearing to be affected was the oxidation of lactate to pyruvate in brain, the inhibition due to chloretone being developed rapidly after its addition.

Jowett[5] reported the inhibition by narcotics of glucose oxidation by slices of cerebral cortex. Also Michaelis and Quastel[6] studied the effect of chloretone on the oxidation of glucose, lactate, and pyruvate by brain tissue. The results suggested that chloretone affects largely either a special flavoprotein or some component of the respiratory

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system of the tissue that plays an intermediate role in the transport between flavoprotein and cytochrome oxidase.

The effect of several drugs on the metabolism of rat brain slices and homogenates was studied by Rosenberg *et al.*[7] By using chloretone they obtained 50 per cent inhibition of oxygen uptake, which they interpreted as indicating an inhibition of Pasteur's effect.

Etling[8–10] found that chloretone inhibited the dehydrogenation of lactic acid by rat brain homogenate but had no influence on the action of glycolytic enzymes; however, it inhibited the oxidation of hexosemonophosphate in brain tissue.

Measurements of respiration and adenosine triphosphate (ATP) were made by Grenell *et al.*[11] on rat brain homogenates subjected to the action of chloretone and sodium pentobarbital. Concentrations of chloretone that would presumably exist in the tissue during a period of effective narcosis did not inhibit either the resting rate of oxygen uptake or the rate of net synthesis of ATP, but at high concentrations severe effects on both could be seen. Such concentrations, however, were considerably beyond those from which any reasonable interpretation of narcotic action could be made.

Ganguli *et al.*[12] have shown that chloretone inhibits oxidation of pyruvate by brain tissue under aerobic conditions and also that there is a relative decrease in phosphorylation. In mitochondrial preparations from guinea pig kidney, Mager and Avi-Dor[13] observed an inhibition of the activity of pyridine nucleotide-dependent oxidases by chloretone. In the nonmitochondrial heart muscle preparation, chloretone blocked the reduced NAD oxidase system at the stage of cytochrome c reductase.

Morikawa *et al.*,[14] on the other hand, found that chloretone caused a slight increase at 5×10^{-4} M and an inhibition of 4·5% at a concentration of 10^{-3} M on the respiration of rat brain cortex slices.

The effect of chlorobutanol on the normal metabolism of animal tissue thus has been studied on a number of biological systems, including intact tissues and different enzymatic activities. However, almost nothing was known of the effect of this compound on the normal properties of mitochondria. It is therefore the purpose of the present paper to show the results of this drug on the respiratory mechanism of heart, cerebrum, and cerebellum slices, and on homogenates and mitochondria.

MATERIAL AND METHODS

Preparation of mitochondria. Rat cerebrum, cerebellum, and heart mitochondria were prepared in a mannitol–sucrose medium, according to the method of Voss *et al.*,[15] containing 0·21 M mannitol, 0·075 M sucrose, 0·01 M Tris, and 0·2 mM EDTA. The final pH was 7·4 White rats, Wistar strain, were decapitated and exsanguinated, and the cerebrum, cerebellum (excluding pons and medulla, and free from the white matter as much as possible), or heart was removed and washed in cold extraction medium and minced. After the homogenization in a Potter-Elvehjem homogenizer, the suspension was centrifuged at 1,000 *g* for 10 min. The supernatant was centrifuged twice again at 10,000 *g* for 10 min. The sedimented mitochondria was finally suspended in the same medium with 0·3 to 0·5 ml/cerebrum, 0·1 ml/cerebellum, or 0·3 ml/heart. The average protein content of these suspensions was equal to 10, 13 and 14 mg/ml for cerebellum, cerebrum, and heart respectively. It must be stated, however, that cerebral and cerebellar mitochondrial preparations can be considered 'mitochondrial fractions.' Further studies of the preparation of the different

subcellular fractions from cerebrum and cerebellum are being carried out in this laboratory, always with electron microscopy as control. The preparation of cerebral mitochondria here described was stable for several hours. Other such preparations have been devised, including the one by Aldridge[16] in which rat brain mitochondria was prepared in 0·3 M sucrose.

Preparation of tissue slices. Tissue slices were obtained by a special technique described by Vianna.[17] The piece of tissue or organ is kept cool by the circulation of cooled water in a special apparatus, and the thickness of the slices can be controlled by a micrometric screw. The tissue slices were placed in cooled Ringer-phosphate and continually aerated; they were then washed twice. Ringer-phosphate was prepared according to Krebs and Henseleit,[18] modified by Barron *et al.*[19] as follows. Ringer: 0·154 M NaCl, 876 ml; 0·154 M KCl, 26 ml; 0·11 M $CaCl_2$, 10 ml; and 0·154 M $MgSO_4$, 4 ml. To 80 ml of this solution 20 ml of phosphate buffer, pH 7·48 (according to Sørensen) was added.

Preparation of homogenates. Rat cerebrum, cerebellum, and heart homogenates were prepared in a medium containing mannitol 0·25 M; Tris 0·01 M; KCl 0·01 M; EDTA 0·2 mM; and phosphate buffer (Sørensen) 0·005 M, pH 7·0. After the homogenization in a Potter-Elvehjem homogenizer, the suspension (5 ml of medium/cerebrum, 2 ml/heart, and 2 ml/cerebellum) was centrifuged at 1,000 *g* for 10 min. The supernatant was used as the homogenate for Warburg respirometry.

Determination of respiration and oxidative phosphorylation. Respiration and oxidative phosphorylation were measured by the sensitive method of the oxygen electrode. The polarograph used was described by Voss *et al.*[20] of this laboratory. It was built with a stationary type of platinum electrode coated with a dialysis tubing paper, the calomel reference electrode being connected to the platinum electrode by a KCl bridge. Being adapted with a magnetic stirrer, the reagents are promptly mixed in a closed mixing chamber. This device has the merit of avoiding the inconvenience of the open cuvets, and by a special setup the temperature can be kept constant. The respiration rates are calculated as $\mu M\ O_2 \cdot sec^{-1} \cdot liter^{-1}$, and the P/O ratios are calculated as ADP/O ratios, by the addition to the system of a known amount of the inorganic phosphate acceptor, according to the methd of Chance and Williams.[21] When the inorganic phosphate acceptor is all consumed, the respiration rate levels off to the control state of respiration after ADP. The ratio between the rate of respiration during ADP and the rate of respiration after ADP gives the value for the respiratory control coefficient (RC). The analysis of the RC gives a numerical value that permits distinguishing a tightly coupled preparation of mitochondria from a loosely coupled one when the controlled respiration with ADP is impaired by the action of different agents, including those that uncouple or inhibit oxidative phosphorylation. An account of the kinetics of oxygen utilization in oxidative phosphorylation is given by Chance and Williams,[22] who discuss the principles of the polarographic method herein described. It is important to know that Chance and Williams have established that the ADP/O values found for mitochondrial preparations correspond to the P/O ratios, since negligible ADP is utilized in reactions other than oxidative phosphorylation.

Determination of the steady-state levels of cytochrome b. Steady-state levels of cytochrome b from heart mitochondria were measured by the use of a two-wavelength technique described by Chance.[23] The instrument used in this laboratory was modified by Cowles.[24] This technique consists essentially in measuring the transmission

TABLE 1. EFFECT OF CHLOROBUTANOL ON THE RESPIRATION OF TISSUE PREPARATIONS

System (A) *for slices*: 2·3 ml of Ringer-phosphate (pH 7·4), 0·2 ml of solution containing 10 μmoles of either succinate or glutamate, 0·2 ml of a solution containing 5·6 μmoles of chlorobutanol and 0·15 ml of KOH 20%. (B) *For homogenates*: 2·3 ml of homogenate (pH 7·0), 0·2 ml of solution containing 10 μmoles of succinate or glutamate, 0·2 ml of a solution containing 5·6 μmoles of chlorobutanol, 0·15 ml of KOH 20%. Volume, 2·85 ml; temperature, 37°, equilibrium time, 10 min; 90 to 100 shakings/min. The average protein content was equal to 1·04 mg/100 ml for cerebrum, 0·294 mg/100 ml for cerebellum, and 1·05 mg/100 ml for heart, all in the original suspension. (C) *For mitochondria*: see Fig. 1.

	Heart			Cerebrum			Cerebellum		
	Substrate	Addition of chlorobutanol	Inhib. (%)	Substrate	Addition of chlorobutanol	Inhib. (%)	Substrate	Addition of chlorobutanol	Inhib. (%)
					Slices (QO_2)				
Endogenous	4·82	3·54	25·0	7·08	5·61	20·0	5·91	3·35	43·0
Succinate	11·80	9·75	17·5	12·40	11·11	10·6	8·14	7·15	12·0
Glutamate	5·67	4·58	19·0	9·13	8·92	2·5	7·15	3·81	46·8
Pyruvate	6·42	5·50	14·0	12·11	8·78	27·6	10·77	8·41	22·0
Glucose	6·42	5·43	15·0	10·09	8·13	19·4	11·79	7·98	32·2
					Homogenates (μlO_2)				
Endogenous	164	141	13·0	173	137	20·8	111	76	31·5
Succinate	344	285	17·0	325	247	24·0	259	207	20·0
Glutamate	249	174	30.0	243	182	25·0	222	103	53·6
					Mitochondria (μlO_2)				
Endogenous	19	15	23·0	75	55	26·6	56	43	23·0
Succinate	244	227	6·8	204	182	10·6	153	143	6·6
Glutamate	156	59	61·2	141	116	17·6	96	80	16·6

changes at two appropriately chosen wavelengths which are specific for each component of the respiratory chain. The effect of chlorobutanol was measured on the basis of reoxidation of reduced cytochrome b from the steady-state levels to which cytochrome b was taken after the appropriate addition of different substrates—α-ketoglutarate, succinate, and glutamate.

Oxygen uptake was measured by the classic Warburg technique. Protein determination from homogenates and mitochondria were done by the micro-Kjeldahl method; dry weights were taken in the usual way.

RESULTS

Effect of chlorobutanol on the respiration of tissue preparations

Respiration of heart, cerebral, and cerebellar tissue slices was studied in classical Warburg experiments with different substrates, with regard to the effects of chlorobutanol added to respiring systems in Warburg respirometers. It was obvious from the beginning that chlorobutanol has a pronounced effect on the endogenous respiration of heart and brain. Table 1 shows the results obtained for slices, homogenates, and mitochondria upon the addition of chlorobutanol, indicating how difficult it is

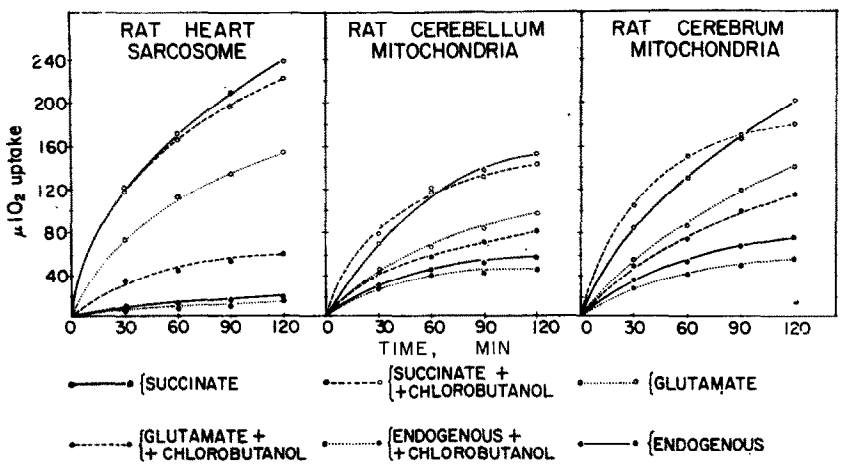

FIG. 1. Effect of chlorobutanol on the oxygen uptake of mitochondrial preparations. Systems: 1·8 ml of medium containing mannitol 0·25 M, Tris 0·01 M, KCl 0·01 M, EDTA 0·2 mM, and phosphate buffer 0·005 M (pH 7·4); 0·5 ml suspension of either heart or cerebral mitochondria; 0·2 ml of solution containing 10 μmoles of succinate or glutamate; 0·2 ml of a solution containing 5·6 μmoles of chlorobutanol and 0·15 ml of KOH 20%. For cerebellar mitochondria the system was: 1·3 ml of medium; 1·0 ml suspension of mitochondria; 0·2 ml of solution containing 10 μmoles of succinate or glutamate; 0·2 ml of a solution containing 5·6 μmoles of chlorobutanol and 0·15 ml of KOH 20%. Volume, 2·85 ml; temperature, 37°; equilibrium time, 10 min; 90 to 100 shakings/min. The average protein content was equal to 2·80 mg/100 ml for heart, 2·41 mg/100 ml for cerebrum, and 1·52mg/100 ml for cerebellum in the original suspension.

to draw conclusions about the effect of such an inhibitor as chlorobutanol on the respiratory activity of tissue slices.

Experiments with homogenates and mitochondria allowed a more direct action of chlorobutanol on the different enzyme systems, thus giving more information on the possible mechanism of action of this drug. Fig. 1 shows the effect of chlorobutanol

on heart sarcosomes and on the mitochondrial fraction of cerebrum and cerebellum. Glutamate respiration by heart and cerebellum homogenates is highly sensitive to the action of chlorobutanol, whereas in cerebrum homogenates it is not inhibited by chlorobutanol; succinate respiration is very little inhibited. Glutamate respiration by heart sarcosomes is shown to be very sensitive to the action of chlorobutanol, which is not the case for cerebrum and cerebellum. Succinate respiration, as shown in the case of homogenates, is not sensitive to the action of chlorobutanol.

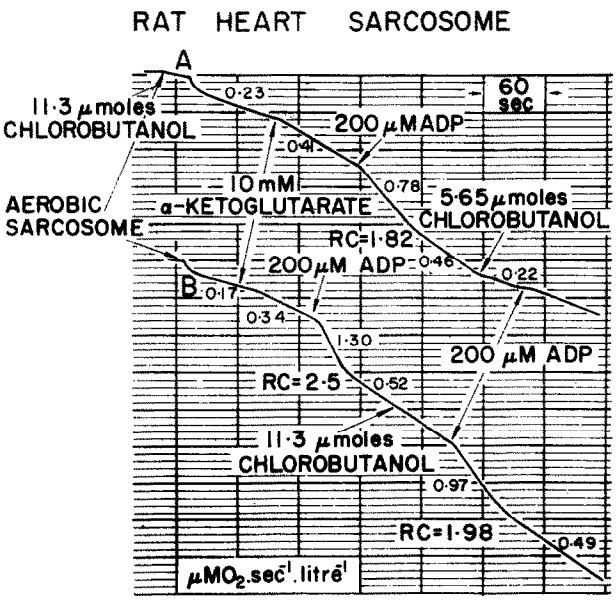

FIG. 2. Effect of chlorobutanol on the normal properties of rat heart sarcosomes. System: 2·2 ml of aerobic medium containing mannitol 0·25 M, Tris 0·01 M, EDTA 0·2 mM, KCl 0·01 M, and 0·005 M phosphate buffer (pH 7·4); 0·2 ml of a suspension of rat heart sarcosomes containing 14 mg of protein per ml. For a description of the experiment see text.

Effect of chlorobutanol on the normal properties of mitochondria

The effect of chlorobutanol on rat heart sarcosomes was assayed polarographically by the method of the oxygen electrode. Figure 2 shows rat heart sarcosomes in systems preincubated and not preincubated with chlorobutanol. In record B, aerobic mitochondria was added with 10 mM α-ketoglutarate, and the rate of oxygen uptake determined (0·34 μM $O_2 \cdot sec^{-1} \cdot liter^{-1}$). This was followed by the addition of 200 μM ADP, which increased the rate of oxygen to 1·30 μM $O_2 \cdot sec^{-1} \cdot liter^{-1}$. When the ADP added was consumed, the rate of oxygen uptake leveled off to 0·52 μM $O_2 \cdot sec^{-1} \cdot liter^{-1}$. The respiratory control coefficient, calculated as the rate between the respiration during ADP and after ADP, gave a value of 2·5. The addition of 11·3 μmoles of chlorobutanol was followed by the addition of 200 μM ADP, which then gave an RC equal to 1·98, an indication that chlorobutanol uncouples the respiratory

chain. In record A, mitochondria was preincubated with 11·3 μmoles of chlorobutanol. The addition of 10 mM α-ketoglutarate showed that the respiration rate was unaffected. However, the addition of 200 μM ADP was followed by a stimulation of the respiration and the consequent leveling off after ADP was consumed, giving a respiratory control coefficient equal to 1·82, which is comparable to the value shown in record B. The further addition of 5·65 μmoles of chlorobutanol, then, inhibited the respiration rate, bringing it to the endogenous level.

Increasing amounts of chlorobutanol have almost no effect on the rate of endogenous and substrate respiration of mitochondria, a fact shown only in high concentrations of chlorobutanol (Table 2). However, there is a clear effect of chlorobutanol on the respiratory control coefficient, which is an indication that the oxidative phosphorylation is increasingly inhibited after the uncoupling of the mitochondria. Cerebellar mitochondria, on the other hand, incubated with glutamate as a substrate, is very sensitive to the addition of chlorobutanol, being completely inhibited by 5·65 μmoles of chlorobutanol. It can be seen then that relatively high concentrations of chlorobutanol are necessary to produce inhibition of the respiratory control ($8{\cdot}99 \times 10^{-4}$ M chlorobutanol inhibits the respiratory control by 25%). In the case of 2,4-dinitrophenol, 2×10^{-4} final concentration is sufficient to uncouple completely oxidative phosphorylation. However, it is important to know whether chlorobutanol can or cannot be considered as an uncoupler of oxidative phosphorylation in the sense that uncouplers such as 2,4-dinitrophenol[25] are able to stimulate the respiration when acting upon the mitochondria. The analysis in Table 2 shows that increasing amounts of chlorobutanol decrease respiratory rate during ADP phosphorylation. A similar fact was observed with thyroxin.[26] This hormone can hardly be considered an uncoupler; added to mitochondria in suitable proportions it inhibits oxidative phosphorylation without showing any stimulating effect.

Action of chlorobutanol on the uncoupling effect of 2,4-dinitrophenol

A polarographic experiment was carried out in order to see the possible effect of chlorobutanol on the property which 2,4-dinitrophenol displays in stimulating the respiration of mitochondria; Fig. 3 shows the results of this experiment.

Effect of chlorobutanol on the steady-state levels of cytochrome b

These experiments were done in order to study the site of action of chlorobutanol on the respiratory chain, since the previous experiments gave an indication that the inhibitory effect of chlorobutanol was somewhat related to the respiratory chain. The experiments were carried out by double-beam spectroscopy. α-Ketoglutarate, succinate, and glutamate were used as substrates for the respiration of mitochondria. The specific pair of wavelengths, 564–575 mμ (for the cytochrome b component), was chosen for these experiments.

The results obtained in three different experiments are shown in Fig. 4. The top (left) record shows an experiment in which the substrate was glutamate; in the top (right) record succinate was the substrate; in the bottom record it was α-ketoglutarate. To the aerobic mitochondria, 15 mM concentration of the corresponding substrate was added to each experiment. The deflection of the inscribing needle indicates the reduction of cytochrome b to the steady-state level. The addition of different amounts of chlorobutanol caused reoxidation of cytochrome b when α-ketoglutarate or glutamate

TABLE 2. EFFECT OF CHLOROBUTANOL ON THE NORMAL PROPERTIES OF MITOCHONDRIA

Chlorobutanol (μmoles)	Endogenous	Substrate respiration	Respiratory rate during ADP (A)	Respiratory rate after ADP (B)	Respiratory control coefficient (RC) (A/B)	Inhibition of RC coefficient (%)
			Rat Heart Sarcosome			
None	0·25	0·41	1·30	0·52	2·50	
2·26	0·22	0·41	1·12	0·60	1·86	25·7
5·65	0·22	0·46	0·98	0·56	1·75	30·0
11·30	0·26	0·49	0·78	0·72	1·08	56·4
16·95	0·24	0·30	0·30	0·30	completely inhibited	100·0
22·60	0·17	0·20	0·20	0·20	completely inhibited	100·0
			Rat Cerebellum Mitochondria			
None	0·12	0·25	0·60	0·30	2·0	
2·26	0·10	0·24	0·60	0·30	2·0	
5·65	0·09	0·21	0·49	0·34	1·2	40·0
11·30	0·07	0·16	0·22	0·22	completely inhibited	100·0
16·95	0·03	0·08	0·08	0·08	completely inhibited	100·0
22·60	0·03	0·06			completely inhibited	100·0

System: 2·2 ml of aerobic medium containing mannitol 0·25 M, Tris 0.01 M, EDTA 0·2 mM, 0·005 M phosphate buffer (pH 7·4), KCl 0·01 M and 0·2 ml of a suspension of mitochondria containing 14 mg protein/ml for heart and 10 mg for cerebellum; the substrate used for heart was α-ketoglutarate (10 mM); for cerebellum, glutamate (10 mM).

was the substrate. In the case of succinate, however, chlorobutanol did not act promptly on the reoxidation of cytochrome b. In the case of glutamate or α-ketoglutarate the further addition of succinate to the inhibited mitochondria causes again the reduction of cytochrome b.

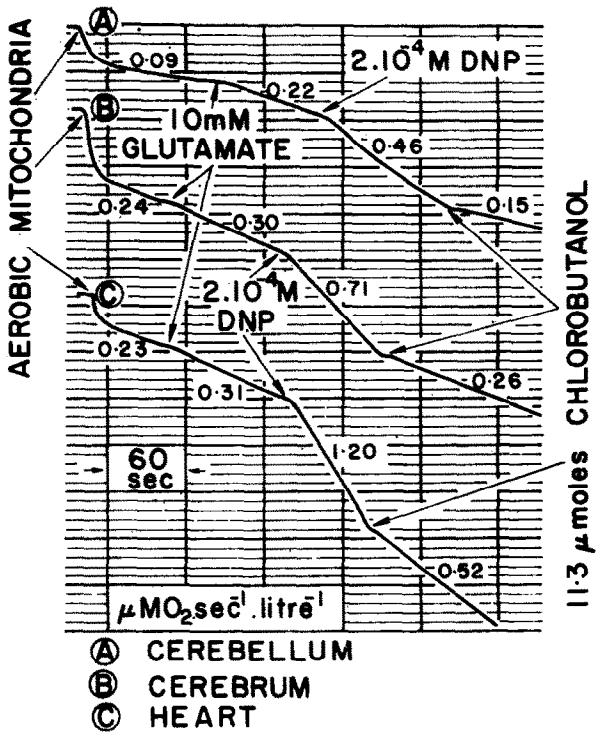

FIG. 3. Effect of chlorobutanol on the uncoupling properties of 2,4-dinitrophenol. System: 2·2 ml of medium containing mannitol 0·25 M, Tris 0·01 M, EDTA 0·2 mM, KCl 0·01 M, and inorganic phosphate 0·005 M (pH 7·4); 0·2 ml of aerobic mitochondria. Aerobic mitochondria of cerebellum (A), cerebrum (B), and heart (C) were added with 10 mM glutamate, the respiratory rates recorded and then added with $2 \cdot 10^{-4}$ M DNP. The addition of 2,4-DNP was followed by a stimulation of the respiration (0·46 μM $0_2 \cdot sec^{-1} \cdot liter^{-1}$ for cerebellum, 0·71 for cerebrum, and 1·20 for heart). The addition of 11·3 μmoles of chlorobutanol caused the inhibition of this effect in every case.

DISCUSSION

The explanation of the effect that chlorobutanol could display on the metabolic properties of animal tissues seems to be contradictory on the basis of the experimental results already found by several investigators. Thus, while Quastel and Wheatley[2, 3] and Jowett[5] showed that the addition of narcotics to cerebral cortex *in vitro* decreased the QO_2, Westfall,[27, 28] Homberger *et al.*,[29] Morikawa *et al.*,[14] Kozawa,[30] and Ito[31] reported the stimulating action of narcotics on the metabolic activity of brain cortex. On the other hand, Rosenberg *et al.*[7] suggested that chlorobutanol inhibits Pasteur's effect, while Etling[8–10] explained the inhibitory effect of this compound by the inhibition of enzymes of the brain glycolytic pathway.

Chlorobutanol shows a clear effect on the endogenous respiration of heart, cerebrum, and cerebellum slices, as well as on the respiratory activity of homogenates and isolated mitochondria. Slices of cerebellum were always more sensitive to the effect of chlorobutanol than were heart and cerebrum, which may be explained on the grounds of its permeability to the drug. In fact, cerebrum slices were apparently less sensitive to the effect of chlorobutanol than cerebellum. It was also possible to show that in different preparations chlorobutanol displayed a clear effect on the glutamate respiration, while succinate respiration was not very much affected by it.

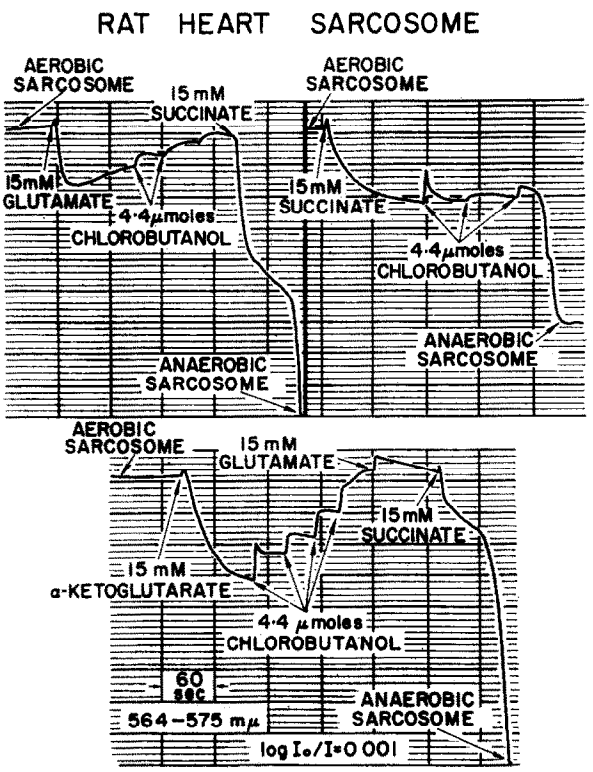

FIG. 4. Effect of chlorobutanol on the steady-state levels of cytochrome b. In the top (left) record the addition of glutamate gave a reduction of cytochrome b which upon addition of chlorobutanol was reoxidized. The further addition of succinate caused again a reduction of cytochrome b. The top right records a similar experiment with succinate as the substrate, showing that the addition of chlorobutanol had no effect on the steady-state levels of cytochrome b. In the bottom record, the addition of α-ketoglutarate and chlorobutanol showed a result very similar to the one displayed by glutamate; i.e. successive additions of aliquots of chlorobutanol caused the reoxidation of cytochrome b. The average protein content was equal to 6·5 mg in 3 ml of the system containing mannitol 0·25 M, Tris 0·01 M, EDTA 0·2 mM, KCl 0·01 M, inorganic phosphate 0·005 M (pH 7·4); 0·2 ml of aerobic mitochondria.

It is known that in cerebrum and cerebellum, glutamate is first decarboxylated to γ-aminobutyrate by the glutamate decarboxylase described by Roberts and Frankel.[32] γ-Aminobutyrate is, then, metabolized to succinic semialdehyde which subsequently goes to succinate. Bacila *et al.*[33] have shown that glutamate respiration in cerebrum

and cerebellum is sensitive to both amytal and malonate, the malonate inhibition being explained by the succinate pathway of respiration, and the amytal inhibition by the NAD-dependent reaction through which succinic semialdehyde is oxidized to succinic acid. Thus the metabolism of glutamate by cerebral and cerebellar mitochondria seems to be mainly through the succinic acid pathway. It has also been shown[33] that γ-aminobutyric acid (GABA) cannot be metabolized directly by these types of mitochondria, as has been suggested by Sacktor *et al.*[34] for brain mitochondria; it is metabolized only when α-ketoglutarate is also present, through the GABA-α-ketoglutarate transaminase, which gives succinate and glutamate. These findings are also in accordance with the results of Voss *et al.*[15] from which it has been found that the ADP/O ratios for succinate and glutamate by cerebral mitochondria are very similar, suggesting that the main pathway of glutamate oxidation by cerebrum is the succinate and not the α-ketoglutarate pathway.

These results might explain the fact that in the present study glutamate oxidation was not greatly affected by chlorobutanol in cerebrum preparations, since this also occurs in the preparations to which succinate is added. In the latter case, the inhibition caused by chlorobutanol was small, compared with the inhibition of respiration of other substrates. The inhibition of glutamate respiration by chlorobutanol in cerebrum was comparable to the inhibition effected in the succinate respiration. However, in the case of heart, glutamate respiration was very sensitive to the action of chlorobutanol, a phenomenon found in homogenates and mitochondria. There was very little or even no effect of chlorobutanol on heart slices, perhaps owing to permeability problems, but in homogenates and in mitochondrial preparations, the inhibition of glutamate respiration by chlorobutanol was very clear, the same not being true for succinate.

Steady-state levels of cytochrome b from heart mitochondria, followed by double-beam spectroscopy, showed that glutamate respiration was very sensitive to the effect of amytal, which also occurs with α-ketoglutarate.[33] It has also been shown that chlorobutanol added to a suspension of heart mitochondria causes the reoxidation of cytochrome b when either α-ketoglutarate or glutamate is the substrate, while the addition of chlorobutanol to the suspension of mitochondria containing succinate does not cause the same effect.

Perhaps as a consequence of this effect, the inhibition caused by chlorobutanol to the normal properties of mitochondria could also be explained. On the other hand, the effect displayed by chlorobutanol in inhibiting the stimulated respiration of mitochondria by 2,4-dinitrophenol suggests that the site of action of chlorobutanol is linked to a phosphorylating step of the respiratory chain. A similar effect has been studied by Ghosh and Quastel.[35] They have found that the addition of chlorobutanol to brain slices has the effect of inhibiting the stimulatory effect displayed by 2,4-dinitrophenol on the respiration. A number of compounds had the same effect on the stimulated oxidation induced by 2,4-dinitrophenol. Thus, Aldridge [36]found that trialkyl tins inhibited oxidation stimulated by apyrase, hexokinase, and glucose or 2,4-dinitrophenol, a property displayed by all trialkyl tins, but not tri-*n*-octyl tin. Similarly, Bacila and Medina[37] demonstrated that phenothiazinic compounds also prevent the stimulation of respiration by 2,4-dinitrophenol of isolated guinea pig heart sarcosomes. It is interesting to note that Aldridge[36] also found that such inhibitors of respiration as cyanide, phenylarsenious acid, or diethy tin did not show the

activation of adenosine triphosphatase by 2,4-dinitrophenol. An extensive study was also made by Aldridge and Parker[38] on the effect of barbiturates on the oxidative phosphorylation of liver and brain mitochondria. They found that oxybarbiturates—phenobarbital, amytal, and hexobarbital—inhibited oxidative phosphorylation, as well as the oxygen uptake stimulated by 2,4-dinitrophenol, but not the activity of adenosine triphosphatase which was stimulated by 2,4-dinitrophenol.

The fact that a similar effect is being described for chlorobutanol together with the findings of Michaelis and Hashimoto[39] that, like amytal, chlorobutanol inhibits disphosphopyridine nucleotide—cytochrome c reductase in rat brain homogenate and dog liver mitochondria, and those of Mager and Avi-Dor,[13] that chlorobutanol inhibits the activity of pyridine nucleotide oxidases, seems to indicate that the site of action of chlorobutanol upon the respiratory chain is in a point located between the electron transport and the phosphorylation of adenosine diphosphate.

Thus it is quite probable that chlorobutanol acts as an inhibitor of the respiratory chain between pyridine nucleotide and flavoprotein. Increasing concentrations are able to act more drastically on the mitochondrial structure or even on other sites of the respiratory chain. Recent investigations from this laboratory (Campello, Voss and Bacila; unpublished data) have shown that the addition of chlorobutanol to cerebral mitochondria causes a very peculiar effect on the b component of the antimycin A-treated respiratory chain, which is a possible indication that chlorobutanol might have more than one effect on the properties of isolated mitochondria.

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