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NARCOTICS AND THE INORGANIC AND CREATINE PHOSPHATES OF MAMMALIAN BRAIN

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Several chemical changes in the brain have now been found to be associated with the action of narcotics *in vivo*. (We have adopted a fairly generally used terminology in which depressants of the central nervous system such as barbiturates and chloral are referred to as narcotics.) These chemical changes include a decreased oxygen uptake by the brain in monkeys (Schmidt, Kety, and Pennes, 1945) and in man (Himwich, Homberger, Maresca, and Himwich, 1946) with pentothal. The formation of lactic acid also decreased (Stone, 1938 ; Richter and Dawson, 1948). Also, the level of inorganic phosphate fell during the action of dial or nembutal (in mice ; Stone, 1943), while that of creatine phosphate rose (in mice: Stone, 1943 ; in rats: Le Page, 1946). Levels of adenosine triphosphates were more stable.

This group of changes appears likely to be interconnected biochemically, but to interpret them adequately from this point of view it is desirable to know whether they can be reproduced in the brain or in parts of the brain under defined conditions *in vitro*. In this respect, respiration has been studied a great deal (see for example Quastel and Wheatley, 1932 ; Quastel, 1943) and, as *in vivo*, has been found to decrease during the action of narcotics on brain slices. Observations with respect to lactic acid, however, are in the opposite sense to the finding *in vivo*. Increased lactic acid formation has been reported by Hutchinson and Stotz (1941) and Buchel and McIlwain (1950) to follow the actions of a variety of narcotics at concentrations in which they inhibited respiration *in vitro*.

Changes in phosphates are associated both with respiration and with lactic acid formation in brain. Also, in brain as in other tissues, phosphates are important intermediaries between functional activity and the metabolism which supports such activity (for a recent assessing see McIlwain, 1950). In interpreting the divergent findings with narcotics *in vivo* and *in vitro*, we therefore considered it important to determine the actions of narcotics on the levels of inorganic and creatine phosphates in brain slices *in vitro*. The substances are extremely labile in brain, and greatly change in concentration during the preparation of brain slices. Nevertheless during metabolism under satisfactory conditions *in vitro* their levels are restored to values which approximate to those in the normal brain *in vivo* (McIlwain, Buchel, and Cheshire, 1950). During a previous study of the actions of inhibitors on brain slices (McIlwain and Grinyer, 1950) it was made evident that in understanding their action it was very necessary to determine their concentration in the tissues on which they were acting. We have accordingly commenced this study with a substance whose concentration could readily be determined, and for this purpose chose chloral. The

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action of barbiturates has also been examined. The work has included subsidiary investigations of the levels of chloral reached in brain tissue during its action *in vivo* and *in vitro*.

EXPERIMENTAL

Tissue metabolism; determination of phosphates.—The methods are described fully elsewhere (McIlwain, Buchel, and Cheshire, 1950). A brief description is as follows. Guinea-pigs were used throughout.

An animal was decapitated, the brain removed, and slices about 0.35 mm. thick cut from the cerebral hemispheres. The slices were placed in Warburg vessels containing balanced physiological salines. High concentrations of phosphates were avoided as buffering agents during metabolic experiments. They would increase the difficulties of determining the phosphates of the slices, and also are not part of the normal environment of tissues. Instead, bicarbonate and glycylglycine were used. Glycylglycine-saline contained: NaCl, 0.134M; KCl, 0.0054M; KH_2PO_4, $MgSO_4$, and $CaCl_2$, 0.00134M, and glycylglycine brought to *p*H 7.4 with NaOH, 0.05M. Glycine, treated similarly, replaced glycylglycine in a few experiments. Bicarbonate-saline contained: NaCl, 0.124M; KCl, 0.005M; KH_2PO_4, $MgSO_4$, and $CaCl_2$, 0.00124M; $NaHCO_3$, 0.021M and was gassed with 5 per cent (v/v) CO_2 in O_2. Glucose was used as substrate at 0.012M and L-glutamic acid, neutralized with NaOH, at 0.02M.

In most cases the salines (3 ml.) were contained in conical Warburg vessels of *c.* 20 ml., the slices added to them, and gas changes followed at 37° by the usual procedures for 15–150 minutes. The slices were then removed with a mounted, bent platinum wire, drained and sometimes reweighed, and dropped into extracting fluid. For larger-scale experiments in which creatine phosphate was separated, some six or eight slices (total fresh weight about 0.35 to 0.5 g.) were added to 15 ml. of metabolic medium in a manometric vessel of about 100 ml.

Two methods were used for determining phosphates. (*a*) Calcium-ethanol precipitations, necessitating about 0.5 g. tissue, and affording results for inorganic phosphates, the phosphate of phosphocreatine, and also the creatine of phosphocreatine. (*b*) A method of differential stability, applicable to *c.* 0.1 g. tissue, and determining inorganic phosphate and phosphates with the lability of phosphocreatine, without their chemical separation. The narcotics used were not found to interfere with the phosphate determination.

Determination of chloral.—The method used was an adaptation of that of Adams (1942) for determining chloral in blood. It depended on the Fugiwara reaction in which a pink colour is formed from chloral and pyridine in strong alkali, and measured photoelectrically. We found specimens of pyridine, even after repeated distillation, to give high blank values. They were purified by boiling the pyridine (50 ml.) and 30 per cent (w/v) NaOH (10 ml.) together for 5 minutes, and distilling the pyridine from the mixture.

For the determination, 1 ml. tungstic acid solution (from one part 20 per cent (w/v) Na_2WO_4 and eight parts 0.167N–H_2SO_4) was contained in each of a set of test-tube homogenizers, 0.1–0.3 g. of tissue or tissue slice added to each, and the mixture immediately homogenized and centrifuged. Development and reading of the colour were done rapidly, as described by Adams, except that groups of up to four tubes were treated together: 0.6 ml. of the supernatant from centrifuging was added to a mixture of 0.6 ml. 30 per cent NaOH and 0.2 ml. pyridine in a small glass-stoppered tube, the tubes placed in a vigorously boiling water bath for exactly 1 minute, then in ice-water for 1 minute and ethanol (1 ml.) added. They were read within 10 minutes, using the micro-cells of a Spekker absorptiometer with a yellow-green filter (Ilford No. 605).

Standard solutions of chloral treated in this way showed a linear relation between extinction and added chloral up to readings of 0.6 (about 60 μg. chloral). A given quantity

of chloral examined on different occasions gave variations in readings up to 15 per cent of their value, and a standard chloral solution was accordingly included with each batch of tubes in which colour was developed. Blank determinations in blood were always zero; brain preparations occasionally gave low blank values. Recovery of chloral was assessed by adding known quantities to 0.3 ml. blood, and preparing tungstic acid filtrates from the mixture. This gave the following results: chloral added, 25 μg., and found, 27; added, 50 μg., and found, 46; added, 75, and found, 73.

RESULTS

Phosphates in slices following metabolism in the presence of narcotics

1. *By chemical separation of inorganic and creatine phosphates.*—By this means it has been shown (McIlwain, Buchel, and Cheshire, 1950) that the phosphocreatine of brain slices, which after preparation fell to 10–20 μg./g., rises during aerobic metabolism in glucose salines to stable values of about 45 μg./g. The rise occurs mainly during the first 20 minutes' incubation and does not occur anaerobically. Conversely the very high inorganic phosphate of the brain after death falls during the experiment, and the ratio inorganic phosphate-P/phosphocreatine-P reaches a value of about two, which approximates to the value found *in vivo*.

The presence of chloral or dial during aerobic metabolism tended to prevent or reduce the normal resynthesis of phosphocreatine. This was to some extent correlated with, and could be understood in terms of, the inhibition of respiration caused by the compounds. Thus (Table I) the low concentrations of chloral which did not affect respiration had little or no action on creatine phosphate. To examine this correlation further, many more values were needed and we therefore turned to the other method of phosphate determination, as described below.

2. *Determination by differential stability.*—The majority of investigations here concerned chloral; some values also are recorded with ethylbutylbarbituric acid (soneryl) and diallylbarbituric acid (dial). The substances were examined under a variety of conditions and, in the concentrations at which they affected phosphates,

TABLE I

CHLORAL AND DIAL ON THE PHOSPHATES OF BRAIN SLICES DURING RESPIRATION *in vitro*

Reaction mixture	Respiration (μmol./g./hr.)	Inorganic phosphate (μg.P/g.)	Creatine phosphate (μg. P/g.)
Glycylglycine, glucose	63	113	42.5
Glycylglycine, glucose with *chloral*, 0.2 mg./ml.	64	116	43
Glycylglycine, glucose with *chloral*, 1.5 mg./ml.	33	126	14
Bicarbonate, glucose, and glutamate	—	110	6.8
Bicarbonate, glucose, and glutamate with *chloral*, 0.1 mg./ml.	—	100	6.8
Bicarbonate, glucose, and glutamate with *chloral*, 1.5 mg./ml.	—	115	3.7
Glycylglycine, glucose	68	104	37.4
Glycylglycine, glucose with *dial*, 0.5 mg./ml.	60	191	27
Bicarbonate, glucose	—	95	55
Bicarbonate, glucose with *dial*, 1 mg./ml.	—	149	39

Determinations were by chemical separation, estimating the creatine phosphate after conversion to creatinine. Experimental period: 40 min.

they produced a consistent effect: namely, an increase in the ratio of inorganic phosphate to phosphocreatine or phosphates of comparable lability.

The largest group of control values previously obtained for normal phosphates of brain, following metabolism *in vitro*, were after 75 minutes, with a glucose saline buffered with glycylglycine. Corresponding values in the presence of chloral are recorded in Table II. No consistent change in phosphates was found to be pro-

TABLE II

CHLORAL ON PHOSPHATES DURING RESPIRATION WITH GLYCYLGLYCINE AND GLUCOSE

Concn. chloral (mg./ml.)	Time of metabolism (min.)	Respiration		Inorganic phosphate		Phosphates of lability of creatine phosphate		Inorganic-P / Creatine-P
		Rate with chloral (μmol./hr.)	Inhibition by chloral % of control without chloral in same exp.)	(μg. P/g.)	(As % change from control)	(μg. P/g.)	(As % change from control)	
0	75	68.5 (S.D., 4.8)		95 (S.D., 9)		45.5 (S.D., 9)		2.1
0.1	75	64.3	−6	106	−4	47	+5	2.1
0.2	75	69.5	0	75	−12	52	+11	1.4
0.2	75	73.5	0	86	0	44	−8	2.0
0.4	75	68.5	6	129	+14	32	0	4.0
0.75	75	66	11	96	0	37	−30	2.6
1.5	75	40.6	59	120	0	12	−55	10
1.5	75	36.9	48	112	+11	26	−41	4.3
1.5	15	46.5	30	109	+4	46	−10	2.4
1.5	135 (1st hr. / 2nd hr.)	35.7 / 26.5	45 / 58	124	+49	16	−57	7.8
1.5	195	39	43	113	+14	25	−51	4.5

Phosphates were determined by differential stability. The value in absence of chloral refers to 15 experiments. Percentage inhibition in individual experiments with chloral are calculated from the control value for that particular experiment.

duced by initial levels of 0.1 and 0.2 mg. chloral/ml. The values in almost every case differ from the mean without chloral, by less than the standard deviation from the mean in the control values. The table records also the percentage difference between each individual value with chloral, and the control without chloral in the same experiment. Here also the deviations are small. With these concentrations of chloral there was also no significant change in respiration.

Progressive changes in respiration and phosphates are seen to follow 0.4, 0.75, and 1.5 mg./ml. of chloral. The most marked changes are in rate of respiration and in the level of phosphocreatine, both of which fall by about 50 per cent with 1.5 mg. chloral/ml. When the effect on phosphocreatine of 1.5 mg. chloral/ml. was followed at different times its action was seen to be less at 15 minutes, but to change little if at all between 75 and 195 minutes. The lactic acid formed in the present experiments in the presence of chloral (Buchel and McIlwain, 1950) causes a slow fall in *p*H. This was 0.2 unit or less at 75 minutes, which was the period of metabolism in most experiments. The fall became 0.7 unit at 195 minutes, but separate experiments showed the change in phosphates to be independent of change in *p*H. Inorganic phosphate tended to rise during the experiments in the presence of chloral,

but the change was proportionally much less than that in phosphocreatine. Thus, 15 experiments without chloral gave mean, 95: S.D., 9. Five experiments with 1.5 mg. chloral/ml. gave mean, 116: S.D., 6. The ratio of inorganic/creatine phosphate also changed markedly in the presence of the higher concentrations of chloral. The results with 1.5 mg./ml. for 75 minutes or longer gave a mean ratio of 6.6 (uninhibited, 2.1).

The picture with other buffers was similar (Table III). Most values are available for bicarbonate-glucose. Here 200 μg./ml. chloral had little action, but 750 and

TABLE III

CHLORAL ON PHOSPHATES, DURING RESPIRATION IN VARIOUS MIXTURES

Reaction mixture	Chloral (mg./ml.)	Time of metabolism (min.)	Respiration		Inorganic phosphate		Phosphates of lability of creatine phosphate		Inorganic-P / Creatine-P
			Rate (μmol./g./hr.)	Change by chloral (%)	(μg. P/g.)	Change by chloral (%)	(μg. P/g.)	Change by chloral (%)	
Bicarbonate, glucose	0	75	—	—	115 (S.D., 15)		44 (S.D., 14)		2.6
Bicarbonate, glucose	0.1	75	—	—	97	−11	35	−10	2.8
Bicarbonate, glucose	0.2	75	—	—	122	+18	48	+12	2.5
Bicarbonate, glucose	0.75	75	—	—	110	+4	31	−31	3.5
Bicarbonate, glucose	1.5	75	—	—	123	+16	13	−71	9.5
Bicarbonate, glutamate ..	1.5	75	—	—	190	+24	46	−22	—
Glycine, glutamate	1.5	75	34	−48	173	+8	30	−20	—
Glycylglycine, glutamate ..	1.5	30	43	−49	234	+26	36	−39	—
Glycylglycine, glutamate and glucose	1.5	150	42	−56	116	+14	29	−47	—
Glycine, glucose	1.5	75	24	−61	133	+49	13	−48	10

Experimental details and expression of results: as Table II

1,500 μg./ml. led to a progressive fall in phosphocreatine. A fall in phosphocreatine was also found with glucose plus glutamate in glycylglycine buffer. Values in bicarbonate-glutamate and glycylglycine-glutamate were in the same sense, but the change was less marked.

The two barbiturates examined had effects similar to those of chloral. Table IV shows the effect of a concentration of soneryl which causes a 40–50 per cent inhibition of respiration. This is seen to be associated with an increase in inorganic phosphate and a decrease in phosphocreatine, under the three sets of experimental conditions recorded in the Table. Dial caused comparable changes in phosphates with markedly less effect on respiration.

Phosphate distribution during the action of chloral

The inorganic phosphates of both slice and metabolic medium have been determined before and after metabolism in the presence and absence of chloral. In

TABLE IV

BARBITURATES ON PHOSPHATES, DURING METABOLISM WITH VARYING BUFFERS

Reaction mixture	Barbiturate (mg./ml.)	Respiration		Inorganic P		Creatine phosphate	
		Rate (μmol./g./hr.)	Change (%)	μg./g.	Change (%)	μg./g.	Change (%)
Glycylglycine, glucose ..	Soneryl 0.3	37	−39	90	+10	24	−46
Glycine, glucose	Soneryl 0.3	30	−50	137	+54	16	−40
Bicarbonate, glucose ..	Soneryl 0.3	—	—	127	+11	29	−33
Glycylglycine, glucose ..	Dial, 0.25	62	−6	127	+3	26	−35
Glycylglycine, glucose ..	Dial, 1.0	52	−21	157	+27	<10	−80 or more

All experiments aerobic; time of metabolism, 75 min.

the absence of chloral, a little phosphate was lost from slice to solution during incubation, and the same has been found to occur in the presence of chloral. After 75 minutes' respiration in glycylglycine saline with glucose as substrate, and 100 or 200 μg. chloral/ml., inorganic phosphate in the medium rose from 33.5 to 37.2 and 37.9 μg./ml. (weight of slices: 90 and 76 mg./3 ml. saline). This was almost the same as the change in the absence of chloral. After 75 minutes' respiration in bicarbonate saline with glutamate as substrate, and 1.5 mg. chloral/ml., the change was from 33.5 to 39 μg. P/ml. (weight of slices: 127 mg./3 ml.), again comparable to that in the absence of chloral. Chloral is not, therefore, causing any major diversion of phosphate metabolism.

Concentration of chloral in brain tissues during its action in vivo *and* in vitro

During action in vivo.—The active dose of chloral in guinea-pigs was found to be very close to that in rats, which had previously been studied in detail (Olszycka, 1938). Doses of chloral were chosen which (Table V) (a) just caused drowsiness and inactivity, and (b) resulted in complete inactivity for about 75 minutes, though the animals responded to touching the eye or to firm pressure on a limb. Those doses, given intraperitoneally, are seen to yield concentrations of chloral of about 60 and about 100 μg./g. respectively, in the brain during their action. The concentration in the blood was approximately the same. No marked variation in concentration was found in the different parts of the brain examined, though little separation could be attempted here, as about 0.3 g. was required for analysis. Duplicate specimens of the same portions of the brain agreed well. The results are in general agreement with those of Archangelsky (1901) who with rabbits and dogs found comparable concentrations of chloral during narcosis. A large measure of uniformity in the distribution of chloral in the brain of dogs was also reported by Veit and Vogt (1935) and the levels of chloral which can be derived approximately from the data of these authors are also of the same order as those in Table V.

During action in vitro.—After metabolic experiments lasting 1 to 1½ hours at 37° and *p*H about 7.4 some 25% of added chloral has been found to be lost. This reaction is associated with acid formation and the displacement of gas from bicarbonate-containing solutions. We have not investigated it further. The bulk of

TABLE V

CHLORAL IN BLOOD AND BRAIN OF GUINEA-PIGS AFTER INTRAPERITONEAL INJECTION

Dose (μg./g. body wt.)	Time after injection	Concentration in (μg./g. or /ml.)				State of animals
		Blood	Cerebral cortex	Subcortical white matter	Cerebellum	
200	5	60	52	47	72	Slightly drowsy
200	10	—	—	—	—	Half asleep
200	55	—	—	—	—	Very drowsy
200	125	—	—	—	—	Normal
225	3–5	—	—	—	—	Progressively more inert
225	5	90	98	94	94	Lying inert
225	14	90	82	82	80	Lying inert
225	37	95	80	65	90	Lying inert
225	75	—	—	—	—	Begins to stir
225	100–150	—	—	—	—	Standing but less active than normal

Chloral administered as a 10% aqueous solution.

the added chloral, which remained at the end of the experiment, was distributed in approximately equal concentrations in the slices and in the solution. This is shown in the different experiments of Table VI. These were carried out with concentrations of chloral which were close to or just less than those causing inhibition of respiration, and these concentrations are immediately seen to be greater than those found in the brain during narcosis.

TABLE VI

CHLORAL IN BRAIN SLICES AFTER METABOLISM IN VITRO

Chloral added (μg./ml.)	Slice wt. (mg. in 3 ml.)		Conditions of metabolism		Chloral finally		
					In soln. (μg./ml.)	In slice	
	Initial	Final	Medium	Period (min.)		μg./g. initial wt.	μg./g. final wt.
200	109	138	Glycylglycine, glucose	60	150	205	158
200	100	126	Glycylglycine, glucose	60	154	205	158
400	67	83	Glycylglycine, glucose	60	266	375	300
400	72	95	Glycylglycine, glucose	60	275	395	284
750	65	—	Glycylglycine, glucose	60	—	898	690*
750	86	—	Glycylglycine, glucose	60	—	884	680*
1,500	65	—	Glycylglycine, glucose	60	—	1,430	1,100*
1,500	58	—	Glycylglycine, glucose	60	—	1,430	1,100*
1,000	93	—	Bicarbonate, glucose	75	—	770	590*
1,000	110	—	Bicarbonate, glucose	75	—	750	580*
1,500	63	—	Bicarbonate, glucose	75	—	1,265	980*
1,500	98	—	Bicarbonate, glucose	75	—	1,115	860*

Experiments were aerobic, with guinea-pig brain cortex. Not all slices were weighed at the end of experiments, and the values marked* were calculated from mean increase in slice-weights under the same metabolic conditions in other experiments.

Discussion

An immediate conclusion from our findings is that the inhibition of respiration by narcotics *in vitro* has very different characteristics from that occurring *in vivo*. *In vitro*, levels of inorganic phosphate in brain cortex are raised and those of creatine phosphate are lowered, and these changes are the opposite of those recorded for the whole brain *in vivo*. Opposite changes, it will be recalled, are also found to be caused by narcotics in the level of lactate: a fall *in vivo* and a rise *in vitro*.

The changes *in vitro* appear likely to follow from the depression of respiration. Inhibition of phosphorylation, parallel to inhibition of respiration by phenobarbitone, has been observed in brain homogenates by Eiler and McEwen (1949). We have made similar observations with chloral (Buchel, see Case and McIlwain, 1950). In the slices, concentrations of the phosphates are being observed and not their rates of change. The lowered concentration of energy-rich phosphates such as phosphocreatine, and the rise in inorganic phosphate, can then be understood as sequelae of the depressed respiration. There are individual differences between the different narcotics in the degree of change in respiration and phosphates, which we have not yet studied. Further, Johnson (1941) and Belitzer (1939) have shown that the breakdown products of the energy-rich phosphates can be expected to stimulate lactic acid formation. The increase in lactic acid resulting from the narcotics may thus also be secondary to their inhibition of the phosphorylation normally accompanying respiration.

The concentrations of chloral active *in vivo* have, however, none of these effects when tested *in vitro*. Presumably, therefore, they are acting on some more susceptible entity which is producing the depression of activity observed as narcosis. It is extremely interesting to see that the biochemical changes observed *in vivo* at the lower concentrations of narcotics can be regarded as following from a depressed functional activity of the brain. Such activity appears to be supported by metabolism through the energy-rich phosphates (see for example McIlwain, 1950) and lesser activity could be understood to leave higher levels of creatine phosphate, and to lead to the formation of less inorganic phosphate and free creatine. Lower lactic acid can then follow by the mechanism referred to above, and lower respiration through the observation that it, too, is dependent on the level of inorganic phosphate (Banga, Ochoa, and Peters, 1939; see also Long, 1945). Acetylcholine, which requires adenosine phosphate or a comparable compound for its formation (Nachmansohn and Machado, 1943; Feldberg and Mann, 1946) also accumulates during narcosis (Richter and Crossland, 1949).

This study offers no explanation of how narcotics *in vivo* may cause depression in the functioning of the central nervous system. Analyses for chloral or phosphates *in vivo* and *in vitro* have concerned relatively large portions of the brain and it is possible that a primary inhibition of respiration may occur *in vivo* at a small centre, more susceptible than the brain as a whole. A more direct action on electrical or ionic phenomena is equally possible.

Summary

1. Narcosis of guinea-pigs by chloral is associated with the presence of 60–100 μg. chloral/g. brain. This is close to the concentration of chloral in the blood of the animals at the same time.

2. Chloral caused no changes in the inorganic phosphate and creatine phosphates of slices of guinea-pig brain, respiring *in vitro*, when it was present in the tissue in concentrations of 100–200 μg./g.

3. Higher concentrations of chloral *in vitro*, which gave concentrations in the tissue of 300–1,000 μg./g., inhibited respiration, decreased creatine phosphate, and increased inorganic phosphate.

4. Dial and soneryl also lowered creatine phosphate and raised inorganic phosphate in tissue slices, in concentrations in which they inhibited respiration *in vitro*.

5. The inhibition of respiration caused *in vitro* by narcotics has therefore very different characteristics from that observed *in vivo*, when phosphocreatine is raised and inorganic phosphate lowered. It is concluded that the biochemical changes found *in vivo* in the bulk of the brain are a result of depressed functional activity caused by the narcotic, and not, as has been urged, its cause.

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