are probably due to the presence of a reactive residue such as serine adjacent to the aspartic acid or asparagine residue (Thompson, 1953).

SUMMARY

Aspartic acid is preferentially liberated during hydrolysis of proteins with hot dilute acids but not with concentrated HCl at 37°. The specificity of the reaction and its mechanism are discussed.

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Effects of Nicotine on the Pyruvic Oxidase System in Brain

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The pharmacology of nicotine has been extensively studied, but comparatively little is known about its mode of action in the biochemical sense.

Nicotine, in common with other pyridine derivatives (Dickens, 1936*a*), inhibits the Pasteur effect and retards the enzymic degradation of cozymase (Baker, Fazekas & Himwich, 1938; McIlwain, 1950). It may interfere with coupled phosphorylation (Case & McIlwain, 1951) but does not affect the hexokinase reaction (Fahmy & Walsh, 1954). Nicotine is particularly toxic to nerve cells (Langley & Dickinson, 1889, 1890*a, b*); the toxicity towards living organisms parallels the degree of development of the nervous system (Greenwood, 1890), and the effects on nerve tissue are analogous to those resulting from oxygen deprivation (McIndoo, 1916; Wilson, 1928).

It is well known that nicotine inhibits cellular respiration and, since nerve is uniquely dependent

on carbohydrate (Dickens, 1936*b*) and is unable to metabolize nicotine (Werle & Müller, 1941; Miller & Larson, 1953), it seemed probable to us that the toxicity of nicotine towards nerve cells might be attributable to its ability to block essential steps in the oxidative metabolism of carbohydrate. In the present work we have made an analytical study of the effects of nicotine on the oxidation of pyruvate through the citric acid cycle.

MATERIALS AND METHODS

Substrates. Neutral solutions were prepared freshly as required from pure, crystalline materials. Sodium pyruvate was prepared as described by Price & Levintow (1952).

Homogenates. Glass homogenizers of Potter & Elvehjem's type were used. Tissues (rat-brain cerebral cortex, pigeon cerebral hemispheres or rat-kidney cortex) were homogenized in Krebs's phosphate-saline, with cooling, to give suspensions containing 100 mg. tissue/ml.

Manometric methods. O_2 uptakes were measured in the Warburg apparatus at 37° and pH 7.4. Gas phase, air. Shaking, 100 strokes/min. The centre wells contained 20% (w/v) KOH (0.2 ml.). Substrates were added to the main compartments from the side arms after 30 min. equilibration.

Citrate was determined by the method of Natelson, Pincus & Lugovoy (1948), using a Unicam Quartz Spectrophotometer.

Acetate was determined by the method of Bartley (1953).

EXPERIMENTAL AND RESULTS

Effects of nicotine on respiration of brain tissue with glucose and pyruvate as substrates

Oxygen uptakes by rat-brain homogenate in the presence and absence of nicotine with either glucose (0.02 M) or pyruvate (0.02 M) as substrate are compared in Table 1. The values obtained in different experiments for respiratory inhibition by nicotine are consistent and are constant throughout the experimental period (2 hr.).

The results show that the oxidation of pyruvate is more sensitive towards nicotine than is that of glucose.

Effect of nicotine on the oxidation of succinate in brain

The effect of nicotine on the oxidation of sodium succinate (0.02 M) by rat-brain homogenate was determined in the presence of sodium arsenite (0.0033 M) which, according to Green, Loomis & Auerbach (1948), inhibits oxidation of succinate in

the cyclophorase system beyond the fumarate $\rightarrow$ malate stage, although the actual site of inhibition by arsenite is probably at the later, oxaloacetate or pyruvate stage (Long, 1945). Under our experimental conditions, arsenite (0.0033 M) reduced the oxygen uptake by rat brain with succinate as substrate from 400 to 120 μ l./100 mg. tissue/1.5 hr. Nicotine at 0.0075 M inhibited by 5–6% and at 0.03 M by 7–8%.

These results are in close agreement with those obtained (unpublished work) when succinic dehydrogenase activities of muscle preparations were measured by the triphenyltetrazolium bromide method, previously described (Fahmy & Walsh, 1952).

Effect of nicotine on the oxidation of fumarate to oxaloacetate in kidney

In spite of the fact that brain contains fumarase (Breusch, 1938) and malic dehydrogenase (Green, 1936), oxygen uptake by brain homogenate is not increased by addition of fumarate. Kidney tissue was therefore used.

The effect of nicotine on oxygen uptake by rat-kidney homogenate with sodium fumarate (0.02 M) as substrate was measured in the presence of methylene blue and cyanide, which binds oxaloacetate (Green & Williamson, 1937), thereby isolating the step, fumarate $\rightarrow$ oxaloacetate (Mann & Quastel, 1941).

The results (Table 2) show that nicotine inhibits this system but has less effect than it has on the pyruvic oxidase system as a whole.

Table 1. *Effects of nicotine on glucose and pyruvate oxidation in brain*

Each vessel contained rat-brain homogenate (100 mg. tissue) in Krebs's phosphate-saline, substrate (0.02 M), $\pm$ nicotine, total vol. 2.5 ml.; O_2 uptakes measured at 37° and pH 7.4.

Glucose				Sodium pyruvate			
Expt.	O_2 (μ l.)			Expt.	O_2 (μ l.)		
	Control	Nicotine (0.0075 M)	Inhibition (%)		Control	Nicotine (0.0075 M)	Inhibition (%)
1 1st hr.	109	98	10	6	147	124	16
2nd hr.	96	86	10		110	90	18
2 1st hr.	118	104	12	7	126	108	14
2nd hr.	102	91	11		100	86	14
3 1st hr.	126	112	11	8	136	116	15
2nd hr.	109	98	10		96	82	15
		(0.03 M)				(0.03 M)	
4 1st hr.	120	95	20	9	139	100	28
2nd hr.	100	77	23		115	79	31
5 1st hr.	110	86	22	10	156	105	33
2nd hr.	88	70	21		128	84	34
				11	144	99	31
					110	73	34

Table 2. *Effect of nicotine on oxidation of fumarate to oxaloacetate in kidney*

Each vessel contained rat-kidney homogenate (100 mg. tissue) in Krebs's phosphate-saline, sodium fumarate (0.02 M), NaCN (0.08 M), methylene blue (0.016%), $\pm$ nicotine, total vol. 2.7 ml.; O_2 uptakes measured at 37° and pH 7.4.

Expt.	O_2 (μ l./100 mg. tissue/1.5 hr.)		Inhibition (%)
		Nicotine (0.0075 M)	
1	70	70	0
2	76	75	1
3	72	71	1
4	84	84	0
		(0.03 M)	
5	80	70	12
6	89	77	13
7	73	64	12
8	90	80	11

Table 3. *Effect of nicotine on oxidative decarboxylation of α -oxoglutarate in brain*

Each vessel contained rat-brain homogenate (100 mg. tissue) in Krebs's phosphate-saline, sodium α -oxoglutarate (0.02 M), sodium malonate (0.033 M), $\pm$ nicotine; total vol. 2.6 ml.; O_2 uptakes measured at 37° and pH 7.4.

Expt.	O_2 (μ l./100 mg. tissue/1.5 hr.)		Inhibition (%)
		Nicotine (0.0075 M)	
1	90	86	5
2	96	90	6
3	92	87	5
		(0.015 M)	
4	86	77	10
5	103	92	11
6	110	97	12
		(0.03 M)	
7	100	85	15
8	98	82	16
9	104	86	17
10	106	90	15

Effect of nicotine on the oxidation of citrate to α -oxoglutarate in liver

Nicotine (0.03 M) had no effect on oxygen uptake at 37° and pH 7.4 by pigeon-liver homogenate (brain tissue being unsuitable) with sodium citrate (0.02 M) as substrate in the presence of sodium arsenite (0.0033 M) which serves to isolate the step, citrate $\rightarrow$ α -oxoglutarate (Krebs & Johnson, 1937). Oxygen uptakes of 150–200 μ l./100 mg. tissue/1.5 hr. were obtained.

Effect of nicotine on oxidative decarboxylation of α -oxoglutarate in brain

The effect of nicotine on the step, α -oxoglutarate $\rightarrow$ succinate was determined by measuring oxygen uptake by rat-brain homogenate with sodium α -oxoglutarate (0.02 M) as substrate in the presence of sodium malonate (0.033 M).

The results (Table 3) show that the inhibiting effect of nicotine on this system is of the same order as that on the oxidation of fumarate to oxaloacetate.

Effect of nicotine on citrate synthesis in brain and kidney

The effect of nicotine on the synthesis of citrate from pyruvate and oxaloacetate by rat brain and kidney have been determined in the presence of inhibitors which prevent the metabolism of citrate. Fluoroacetate was used in experiments with kidney. Since brain tissue, unlike that of kidney, does not form the inhibitor from fluoroacetate (Peters, 1953), $BaCl_2$ was used in experiments with brain (Wieland & Rosenthal, 1943).

Mixtures (3.0 ml.), with and without nicotine, containing tissue homogenate ($\approx$ 300 mg. tissue), pyruvate (0.02 M), oxaloacetate (0.02 M) and either $BaCl_2$ (0.033 M) or fluoroacetate (0.01 M) were shaken in the Warburg apparatus at 37° and pH 7.4. Samples (1.0 ml.) were withdrawn at zero time and after 1 hr. for determination of citrate, having heated to destroy citrate-simulating substances as recommended by Krebs & Eggleston (1948). In the experiments with kidney, an initial incubation period (15 min.) was allowed to permit fluorocitrate to be formed before adding the substrates.

The results (Table 4) show that the inhibition (40%) of citrate formation by nicotine (0.03 M) is greater than that of any of the other steps in the citric acid cycle, and that brain and kidney tissue are, in this respect, equally affected.

In experiments carried out on the synthesis of citrate from acetate by baker's yeast, following the procedure described by Ryman & Walsh (1954), nicotine had no appreciable effect, thereby suggesting that nicotine, unlike cocaine, does not inhibit the condensing enzyme.

Effect of nicotine on oxidative decarboxylation of pyruvate

The effects of nicotine on the amount of acetate formed from pyruvate in dialysed pigeon-brain homogenates (Banga, Ochoa & Peters, 1939) have been determined in the system as used by Coxon, Liébecq & Peters (1949). The results (Table 5) show that nicotine inhibits the pyruvic dehydrogenase system to the same extent as it inhibits the synthesis of citrate from pyruvate. It would appear, therefore, that the inhibitory action of nicotine on the

Table 4. *Effects of nicotine on citrate synthesis in brain and kidney homogenates*

Mixtures (3.0 ml.), containing in Krebs's phosphate-saline, tissue (300 mg.), sodium pyruvate (0.02 M), sodium oxaloacetate (0.02 M), either BaCl₂ (0.033 M; brain) or sodium fluoroacetate (0.01 M; kidney), $\pm$ nicotine, were shaken in air at 37° and pH 7.4. Citrate synthesized = 1 hr. value - 0 hr. value.

Expt.	Brain			Kidney		
	Citrate (μ g./300 mg. tissue/hr.)		Inhibition (%)	Citrate (μ g./300 mg. tissue/hr.)		Inhibition (%)
	Control	Nicotine (0.0075 M)		Control	Nicotine (0.0075 M)	
1	189	151	20	870	736	15
2	199	161	19	825	680	18
3	150	122	19	850	697	18
	(0.015 M)			(0.015 M)		
4	149	99	34	900	612	32
5	201	140	30	814	570	30
6	170	119	30	730	508	30
	(0.03 M)			(0.03 M)		
7	179	100	44	680	415	39
8	220	132	40	780	468	40
9	180	108	40	912	538	41

Table 5. *Effects of nicotine and vitamin B₁ on pyruvic dehydrogenase in dialysed pigeon-brain homogenate*

Each flask contained in 0.08 M potassium phosphate buffer (2.5 ml.), tissue (200 mg.), ATP (1 μ mole), MgCl₂ (4 μ moles), sodium pyruvate (30 μ moles), $\pm$ nicotine, $\pm$ vitamin B₁. Acetate formed at 37° and pH 7.4 = 0.5 hr. value - 0 hr. value.

Expt.	Molar concentration of nicotine and/or vitamin B ₁	Acetate (μ g./200 mg. tissue/0.5 hr.)		Activation (%)		Inhibition (%)	
		Control	Nicotine	Vitamin B ₁	Nicotine + vitamin B ₁	Vitamin B ₁	Nicotine + vitamin B ₁
1	0.0075	560	440	590	460	5	21
2		480	400	520	432	8	17
3		510	420	525	430	3	18
4	0.015	600	420	615	425	3	30
5		560	405	565	396	1	29
6		620	415	630	435	2	33
7		510	376	500	355	-2	28
8	0.03	547	322	580	360	6	41
9		501	300	520	302	4	40
10		602	360	600	365	0	40
11		520	300	516	300	0	42

pyruvic oxidase system is the result of a specific inhibition of pyruvic dehydrogenase.

We have included in our experiments a study of the effect of nicotine on this system in the presence of added vitamin B₁. Vitamin B₁ was added to give a final concentration equal to that of the nicotine. The results show that addition of vitamin B₁ does not protect the enzyme from the inhibitory action of nicotine.

It is of interest to report here that addition of vitamin B₁ to homogenates, prepared from the brains of pigeons which were used immediately after purchase, increased acetate formation by as much as 20%. On the other hand, vitamin B₁ had little or no effect when added to homogenates prepared from pigeons which had been kept in our own animal house for several days.

DISCUSSION

Preliminary experiments with yeasts (unpublished data), analogous to those employed in the study of the action of cocaine on cellular metabolism (Ryman & Walsh, 1951, 1952), have confirmed the reports of earlier workers (reviewed by Enders & Windisch, 1947) that nicotine, while inhibiting respiration, activates fermentation and may even accelerate anaerobic growth.

Himwich & Fazekas (1935, 1936) reported that nicotine inhibited respiration of minced brain with lactate or succinate as substrates but not with either glucose or pyruvate. They concluded that lactic dehydrogenase was probably inhibited. Later, Baker *et al.* (1938), using brain slices, found glucose and pyruvate oxidation to be inhibited

while succinate oxidation was little affected. They reported inhibitions of 49 % with glucose, 76 % with pyruvate and 91 % with lactate by nicotine (0.3 M). In our experiments with homogenates of rat-brain cerebral cortex we have not obtained inhibitions as high as those reported by Baker *et al.*, but we have obtained definite and consistent inhibitions of respiration with glucose as substrate and consistently higher inhibitions with pyruvate. These results emphasize that the site of action of nicotine in inhibiting carbohydrate oxidation lies in the metabolic path following the pyruvate stage.

Since inhibition of succinate oxidation had previously been reported, and in view of the possibility that nicotine might depress oxidation, not by inhibiting any of the enzymes specific to the substrates of the citric acid cycle but by interfering with the electron-transferring mechanism of respiration, we investigated the effect of nicotine on the aerobic oxidation of succinate in the presence of arsenite. Nicotine had but little effect on this system, thus indicating that succinic dehydrogenase and its associated cytochrome system are not appreciably affected. This information completed the evidence that nicotine does not inhibit respiration by interference with the general mechanism of hydrogen and electron transfer. Experiments with other dehydrogenase systems (unpublished work) have shown that nicotine is not a general inhibitor of systems involving either cozymase or the flavoproteins.

The experiments with other isolated steps of the citric acid cycle show that nicotine (0.03 M) has no appreciable effect on the oxidation of citrate to α -oxoglutarate, and that the inhibitions of the enzymes involved in the oxidations of fumarate to oxaloacetate and of α -oxoglutarate to succinate are insufficiently great to account for the effect of nicotine on pyruvate oxidation.

While the synthesis of citrate from acetate by baker's yeast is not affected by nicotine, citrate synthesis in brain and kidney homogenates, with pyruvate as the source of active acetate, is inhibited, nicotine (0.03 M) inhibiting by 40 %. It is of interest to note that there is no difference between brain and kidney tissue in the effects of nicotine on citrate synthesis.

The results of investigations into the effect of nicotine on the oxidative decarboxylation of pyruvate, using dialysed brain homogenate to isolate the step, pyruvate $\rightarrow$ acetate, confirm that the inhibitory action of nicotine on pyruvate oxidation may be attributed primarily to a specific inhibition of pyruvic dehydrogenase.

Although nicotine inhibits choline acetylase (Fahmy, Ryman & Walsh, 1954), it has no appreciable effect either on sulphanilamide acetylation in liver or on citrate synthesis from acetate in yeast. It

would appear, therefore, that nicotine is not a general inhibitor of all reactions in which active acetate is involved.

With regard to any discussion concerning the mechanism for the inhibitory action of nicotine on the oxidative decarboxylation of pyruvate, it is relevant to compare the behaviour of this system with that concerned in the analogous oxidative decarboxylation of α -oxoglutarate. As pointed out by Green (1952), both systems require the same cofactors but, as our experiments show, the α -oxoglutarate system is much less sensitive towards nicotine. It is also much less sensitive towards deficiency of vitamin B₁ (Peters, 1953).

Since some of the pharmacological effects of nicotine are antagonized by vitamin B₁ (Unna & Pick, 1944; Jalón & Bayo, 1946; Haimovici & Pick, 1946; Pick & Unna, 1946), and there is some structural resemblance between the two compounds, one might have expected to find a biochemical antagonism between them. The results obtained in the present work, however, show that addition of vitamin B₁ to the pyruvic dehydrogenase system has no influence on the inhibitory action of nicotine when present in equal concentration. We have also observed, in our preliminary experiments with yeasts, that the stimulating effects of nicotine and of vitamin B₁ on fermentation are additive and apparently independent of one another.

It is of interest to note that barbiturates also block aerobic pyruvate metabolism by inhibiting pyruvic dehydrogenase and to a lesser extent the oxidative decarboxylation of α -oxoglutarate (Persky, Goldstein & Levine, 1950).

SUMMARY

1. Nicotine inhibits the oxidation of pyruvate by brain tissue more strongly than that of glucose.
2. Nicotine inhibits pyruvate oxidation in brain by specifically inhibiting pyruvic dehydrogenase, other oxidative steps being less sensitive.
3. Addition of vitamin B₁ does not modify the effect of nicotine on the pyruvic dehydrogenase system in dialysed pigeon-brain homogenate.

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Incorporation of Radioactive Phosphate into Lipids and Pentosenucleic Acid of Cat Brain Slices. The Effect of Inorganic Ions

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It is well known that an increase in the concentration of potassium ions, or a decrease in the concentration of calcium ions, causes an increase in the oxygen consumption and aerobic glycolysis of brain slices. There is also a decrease in the anaerobic glycolysis. More recently, McIlwain (1952a) and Gore & McIlwain (1952) have shown that if the concentration of K^+ in the medium is increased or the concentration of Ca^{2+} is decreased, respiring slices of guinea pig brain are unable to maintain their concentration of phosphocreatine and other labile phosphorus compounds.

Since it has been shown previously that the incorporation of inorganic phosphorus labelled with ^{32}P into the pentosenucleic acid (DeLuca, Rossiter & Strickland, 1953), phospholipid (Strickland, 1954), and other protein-bound phosphorus compounds (Findlay, Strickland & Rossiter, 1954) of respiring brain slices is dependent upon oxidative phosphorylation mechanisms within the slice, it seemed of interest to determine the effect of increasing the

concentration of K^+ or decreasing the concentration of Ca^{2+} on the incorporation of inorganic ^{32}P into these phosphorus-containing compounds. Since the addition of NH_4^+ , L-glutamate, or L-glutamine, also causes a decrease in the concentration of phosphocreatine in respiring brain slices (McIlwain, 1951, 1952a; Gore & McIlwain, 1952), the effect of these substances was also investigated.

A preliminary account of some of these experiments has appeared (Rossiter & Findlay, 1954).

METHODS

Slices of cat brain were incubated in Warburg flasks containing Krebs-Ringer bicarbonate, glucose and $[^{32}P]$ -phosphate. The vessels were filled with a mixture of 95% O_2 and 5% CO_2 and incubated in a bath at 37.2° for 4 hr., with shaking to ensure proper oxygenation. When inorganic ions were added or omitted, the medium was kept at the same tonicity by adding or omitting the requisite quantity of NaCl. Inorganic constituents were added as the sodium salt. Details of the preparation of tissue and incubation have been described by DeLuca *et al.* (1953).