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THE EFFECT OF PIPERAZINE ON SUCCINATE PRODUCTION BY *ASCARIS LUMBRICOIDES*

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Piperazine reduced the production of succinate by *Ascaris lumbricoides*. This effect was reversible. There was a close parallelism between the concentrations of piperazine which paralysed the worm and those which inhibited the formation of succinate. Piperazine did not affect the incorporation of [2-^{14}C]lactate into succinate by strips of *Ascaris* muscle. It was concluded that production of succinate supplies energy for the contraction of *Ascaris* muscle.

Succinic acid has been identified in the perienteric fluid of *Ascaris lumbricoides* (*var. suis*) (Bueding and Farrow, 1956). In the muscle of this parasite at least two active enzymatic mechanisms are available which can account for the utilization of succinate: (*a*) the succinic oxidase system for the formation of fumarate (Bueding and Charms, 1952; Bueding, Entner, and Farber, 1955), followed by its conversion to pyruvate catalysed by fumarase and a "malic" enzyme (Saz and Hubbard, 1957); (*b*) decarboxylation of succinate to propionate (Saz and Vidrine, 1959). The presence of these metabolic systems concerned with the utilization of succinate together with the high concentration of succinate in the body fluid of *Ascaris* (Bueding and Farrow, 1956) suggest that succinate is very rapidly formed. The present paper reports that production of succinate by intact *Ascaris* can be demonstrated readily, a process inhibited reversibly by piperazine. This compound produces paralysis of the worm (Brown, Chan, and Hussey, 1955; Swartzwelder, Miller, and Sappenfield, 1955), and is effective in the treatment of ascariasis (see review by Bueding and Swartzwelder, 1957).

METHODS

The parasites were incubated for 24 hr. at 38° in Erlenmeyer flasks with a modified Ringer-Baldwin medium containing NaCl, 8.0 g.; KCl, 200 mg.; $CaCl_2$, 200 mg.; $MgSO_4$, 400 mg.; $NaHCO_3$, 1.8 g.; 2N-NaOH, 1 ml.; glucose, 2 g.; sulphafurazole, 500 mg.; penicillin, 100 mg.; streptomycin, 75 mg.; nystatin (Mycostatin), 10 mg.; distilled water, 1 l. While in earlier studies the addition of a sulphonamide, penicillin and streptomycin to the medium eliminated contamination with micro-organisms (Epps, Weiner, and Bueding, 1950), in the present experiments fungi grew not infrequently unless nystatin was present. This compound had no effect on the production of succinate by *Ascaris* in the concentration used. The worms were blotted and weighed before their transfer into the incubation medium. Each flask contained three worms in 150 ml. of medium.

The concentration of succinate in the worms was determined as follows. The parasites were homogenized in three times their weight of perchloric acid (5% v/w) and the precipitated proteins were separated by centrifugation at 3,000 rev./min. for 30 min.; a measured volume of the supernatant fluid was neutralized to *p*H 7.0 with 5N-KOH and its total volume recorded; to remove the insoluble potassium perchlorate the mixture was centrifuged (3,000 *g*; 30 min.). These operations were carried out between 2 and 4°. An aliquot of the supernatant was adjusted to *p*H 1.8 with 5N-H_2SO_4 and extracted with ether in a continuous extraction apparatus for 24 hr. The extract was evaporated to dryness, taken up in water, neutralized to *p*H 7.0 with 2N-KOH and the volume brought to 2.0 ml. with water; the succinate content of an aliquot of this neutralized extract was determined enzymatically by the method previously used (Bueding and Farrow, 1956). The succinate concentration of aliquots (usually 60 ml.) of the medium was analysed in the same manner, except that treatment with perchloric acid was omitted.

Stock solutions of piperazine were prepared by adjusting a concentrated solution of piperazine hexahydrate (40%) with 2N-HCl to *p*H 7.4. The incubation medium was used for further dilutions. All concentrations of piperazine are expressed in terms of the weight of the free base.

Ascaris muscle strips were obtained as described by Waters (1954) and incubated with [2-^{14}C]lactate in

a conventional Warburg apparatus. At the end of the incubation period, enzymatic reactions were stopped by placing the incubation mixture in a boiling-water bath for 10 min. After steam distillation, ether extraction and chromatography from Celite, succinate and lactate were isolated (Saz and Vidrine, 1959). The succinate fraction thus obtained was treated with $KMnO_4$ (Friedemann and Graeser, 1933) to oxidize possible contaminating acids. The succinate was re-isolated by ether extraction and again chromatographed from Celite. Succinate was degraded by oxidizing completely an aliquot of the sample to CO_2 (Van Slyke and Folch, 1940). Samples of $^{14}CO_2$ were precipitated as $Ba^{14}CO_3$ and the precipitate was collected as a finite layer on filter paper discs. Samples thus obtained were assayed for radioactivity with an ultra-thin window gas-flow counter.

TABLE I

PRODUCTION OF SUCCINATE BY *A. LUMBRICOIDES VAR. SUIS*

The worms were incubated for 24 hr. at 38° in a modified Ringer-Baldwin medium.

Expt. No.		Succinate Content (μM/10 g. of Worms)			
		Worms	Medium	Total	Change
1	Before incubation	62		62	
	After ,,	48	52	100	+38
2	Before ,,	55		55	
	After ,,	44	65	109	+54
3	Before ,,	55		55	
	After ,,	48	45	93	+38
4	Before ,,	66		66	
	After ,,	72	47	119	+43

TABLE II

EFFECT OF PIPERAZINE ON SUCCINATE PRODUCTION BY *A. LUMBRICOIDES*

Incubation period, 24 hr. Temperature: 38°.

Expt. No.	Piperazine (%)	Succinate Content					
		Worms		Medium		Total	
		(μM/10 g. of Worms)	Change (%)	(μM/10 g. of Worms)	Change (%)	(μM/10 g. of Worms)	Change (%)
1	—	74		38		112	
	0·1	50	−32	2	−95	52	−54
2	—	66		28		94	
	0·1	43	−35	4	−86	47	−50
3	—	86		67		153	
	0·1	68	−21	6	−91	74	−52
4	—	52		58		110	
	0·05	34	−35	5	−91	39	−64
5	—	55		64		119	
	0·05	36	−35	5	−92	41	−65
6	—	69		47		116	
	0·05	48	−31	7	−85	55	−57

RESULTS

Production of succinate by *Ascaris* was demonstrated readily by determination of succinate in the worms and in the incubation medium (Table I). While the succinate concentration of the worms did not change greatly during incubation, large quantities of succinate appeared in the medium; thus, the total amount of succinate found in the worm and in the medium was considerably greater than that present in the parasite before incubation.

While trying to see if piperazine produced any biochemical changes in *Ascaris*, it was found that this anthelmintic markedly reduced the production of succinate (Table II). After incubation with paralysing concentrations of piperazine, the succinate content of the worms was decreased significantly; the reduction in the amount of succinate released into the medium was even more pronounced. Succinate production was inhibited by piperazine to the same extent when glucose was omitted from the medium. This effect of piperazine was reversible: when, after incubation with piperazine, the worms were transferred into piperazine-free medium the total amount of succinate was about the same as in controls not incubated previously with piperazine (Table III).

Fig. 1 illustrates the relationship between the concentration of piperazine and the reduction in the succinate content of the medium. There was a good correlation between the paralysing action of piperazine and its inhibition of succinate production. When paralysis was complete, succinate production was strongly inhibited.

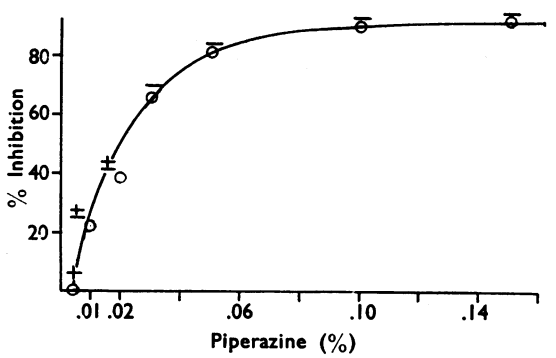

FIG. 1.—The relation between concentration of piperazine and inhibition of succinate production. −, Non-motile; ±, barely motile; +, motile.

Concentrations of piperazine which produced incomplete, yet clearly noticeable, reduction in motility inhibited succinate production appreciably but to a lesser extent. Piperazine in a concentration which did not affect motility did not alter succinate production.

The action of piperazine on succinate production differs in some respects from that of the phenothiazine derivative promethazine (Table IV). In the first place, succinate production was inhibited to a lesser degree than with piperazine. Furthermore, inhibition of succinate production not only persisted but became much more pronounced after the worms had been transferred into medium free of promethazine. Therefore, in contrast to piperazine, promethazine has an irreversible effect on succinate production.

Ascaris muscle strips incorporate [2-^{14}C]lactate into succinate (Saz and Vidrine, 1959). The effect of piperazine upon the incorporation was studied to determine whether paralysis results from an impaired rate of formation of succinate, or *vice versa*. Six worms were incubated in each of two flasks containing the glucose-salt-antibiotic mixture, one with 0.1% piperazine as well. After 24 hr., muscle strips were obtained from both piperazine-treated and untreated worms and incorporation of [2-^{14}C]lactate into succinate was determined. Piperazine (0.1%) was added only to the incubation mixture which contained the muscle strips from piperazine-treated worms. Results of these experiments are recorded in Table V. It can be seen that piperazine had essentially no effect upon the incorporation of [2-^{14}C]lactate

TABLE III

REVERSIBILITY OF THE INHIBITORY EFFECT OF PIPERAZINE ON SUCCINATE PRODUCTION IN *A. LUMBRICOIDES*

After the first incubation period (24 hr.; 38°) the worms were transferred into piperazine-free control media and incubated for another 24 hr.

Expt. No.	Piperazine (%)	First Incubation Period						Second Incubation Period					
		Succinate Content						Succinate Content					
		Worms		Medium		Total		Worms		Medium		Total	
		(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)
1	—	55		64		119		61		72		133	
	0·05	36	−35	5	−92	41	−65	79	+30	62	−14	141	+6
2	—	66		28		94		45		73		118	
	0·1	43	−35	4	−86	47	−50	58	+29	58	−20	116	−2
3	—	78		48		126		66		42		108	
	0·05	43	−45	6	−88	49	−61	61	−8	37	−12	98	−10

TABLE IV

IRREVERSIBILITY OF THE INHIBITORY EFFECT OF PROMETHAZINE ON SUCCINATE PRODUCTION IN *A. LUMBRICOIDES*

After the first incubation period (24 hr.; 38°) the worms were transferred into promethazine-free control media and incubated for another 24 hr.

Expt. No.	Promethazine (%)	First Incubation Period						Second Incubation Period					
		Succinate Content						Succinate Content					
		Worms		Medium		Total		Worms		Medium		Total	
		(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)
1	—	82		47		129		72		42		114	
	0·004	74	−10	38	−19	112	−13	44	−39	5	−88	49	−57
2	—	60		41		101		53		35		88	
	0·004	48	−20	28	−32	76	−25	22	−59	0	−100	22	−75
3	—	47		49		96		43		38		81	
	0·004	41	−13	29	−41	70	−27	21	−51	4	−89	25	−69

TABLE V

EFFECT OF PIPERAZINE UPON THE INCORPORATION OF [2-^{14}C]LACTATE INTO SUCCINATE BY MUSCLE STRIPS OF *A. LUMBRICOIDES*

Worms were incubated in the modified Baldwin-Ringer medium with or without piperazine (0.1%). After 24 hr., muscle strips were obtained from six piperazine-treated and six control worms. Each set of strips was added to a Warburg vessel which contained 150 μmole of lithium [2-^{14}C]lactate (count/min. = 49,760/μmole), 0·1% piperazine where indicated and antibiotic-free medium. Vessels were shaken for 100 min. at 37° ; the gas phase was 95% N_2 and 5% CO_2. The weight of muscle in each flask was 7 to 8 g.

Expt. No.	Piperazine	Lactate Disappearance (μmole)	Succinate Content				
			Recovered	Total Radioactivity		Specific Radioactivity	
			(μmole)	(Count/min.)	Change (%)	Count/min./μmole	Change (%)
1	−	65·2	55·3	184,700		3,340	
	+	56·2	54·9	158,660	−14·1	2,890	−13·5
2	−	−	44·4	144,740		3,260	
	+	−	39·4	147,360	+1·8	3,740	+14·7

into succinate. There was a slight decrease in the specific and total radioactivities in Experiment No. 1, and a slight increase in Experiment No. 2. As these are considered to be within the range of experimental variation piperazine has no effect upon the conversion of lactate to succinate.

DISCUSSION

Norton and de Beer (1957) have reported that piperazine blocks the response of *Ascaris* muscle to stimulation by acetylcholine; the concentration/response curve obtained by these authors is very similar to that relating concentration of piperazine to inhibition of succinate production (Fig. 1). Furthermore, both the neuromuscular blocking (Norton and de Beer, 1957) and metabolic effects of piperazine are reversible. Therefore, the question arises whether and in what manner these two actions of piperazine on *Ascaris* are related. The paralysis may either be the cause or the result of the reduced rate of formation of succinate. It is conceivable that, at the neuromuscular junction, chemical reactions associated with succinate production may be required for the response of *Ascaris* muscle to acetylcholine. If this were so, piperazine should inhibit succinate production in isolated muscle strips from *Ascaris*. However, paralysing concentrations of piperazine do not affect appreciably the rate of incorporation of [2-^{14}C]lactate into succinate. Therefore, it is concluded that the inhibition of succinate production in *Ascaris* by piperazine is the result of myoneural block. In skeletal muscle of vertebrates the energy from the anaerobic conversion of carbohydrate to lactic acid is utilized ultimately for muscular contraction through resynthesis of energy-rich phosphate compounds. In *Ascaris*, reactions involved in the formation of succinate may be concerned with the generation of energy for muscular contraction; if this were so, reduction or complete suppression of muscular activity by piperazine would lower the energy requirement of muscle and result in a decreased formation of succinate. In the light of the findings of Norton and de Beer (1957) the observations reported here indicate that production of succinate plays a rôle in the chemistry of muscular contraction of *Ascaris*.

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