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PHARMACOLOGICAL RESPONSES OF THIAMINE-DEFICIENT RAT TISSUES

BY

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Rats were rendered deficient in thiamine by feeding a synthetic diet free of the vitamin. Responses of the isolated heart to acetylcholine, adrenaline, noradrenaline and isoprenaline, and of the phrenic nerve-diaphragm preparation to tubocurarine, gallamine and eserine, were compared with responses of tissues obtained from littermate rats pair-fed an identical diet with the addition of 25 μg of thiamine hydrochloride per day. In thiamine-deficient tissues eserine failed to produce sustained potentiation of the twitch response of the phrenic nerve-diaphragm preparation to single supramaximal nerve stimuli. The perfused thiamine-deficient heart was more sensitive to acetylcholine, adrenaline, noradrenaline and isoprenaline, which produced greater negative or lesser positive chronotropic and inotropic effects. There was no significant difference in the response of the phrenic nerve-diaphragm preparation to tubocurarine and gallamine, or to eserine with faradic stimulation.

While much effort has been directed to studying the biochemical aspects of thiamine deficiency, the pharmacological responses have remained largely neglected until recently (Bhagat & Lockett, 1962a, b). This is surprising in view of the belief of Muralt (1947) and others (Woolley, 1951 ; Woolley & Merrifield, 1954 ; Naber, Cravens, Baumann & Bird, 1954) that thiamine is involved in nerve metabolism and, in particular, in neuromuscular transmission (Muralt, 1947), and in view of the fact that thiamine pyrophosphate is implicated in acetylcholine synthesis (Birks & MacIntosh, 1957).

The experiments described here were performed in order to study the effects of thiamine-deficiency at the neuro-effector cell junction in an attempt to elucidate the role of thiamine at this site.

METHODS

Rats were rendered deficient in thiamine by feeding a synthetic diet free of thiamine, as described by Boullin (1961) except that 60% purified casein was replaced by sucrose.

Littermate male weanling albino Wistar rats were segregated into pairs of approximately equal weight ($\pm 10\%$). One rat received the deficient diet, and its littermate was pair-fed an identical diet with the addition of 25 μg of thiamine hydrochloride per day. The rats were usually given the experimental diets when four weeks old.

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Recognition of thiamine deficiency. The classical signs of thiamine deficiency include bradycardia and convulsions (Kon & Drummond, 1927; Drury, Harris & Maudsley, 1930; Sandels, 1930). These two signs were used as the essential criteria of the deficiency state, according to the arbitrary classification of Boullin (1961). Animals with a heart rate of 360 beats/min or less were considered to be acutely deficient, whereas those with higher rates (up to 420 beats/min) were considered to have chronic deficiency.

The heart rates were recorded while restraining the rat in a prone position on a wooden board. Spring-clip electrodes were fitted to the legs, protected by cotton wool pads soaked in 0.9% saline. The electrodes were connected to a conventional push-pull AC-amplifier, the output of which was passed into a discriminator circuit adjusted to select the pulses of greatest amplitude. These pulses triggered a flip-flop circuit, whose output was of suitable duration to operate a Thorp impulse counter (C. F. Palmer, London). The counter recorded

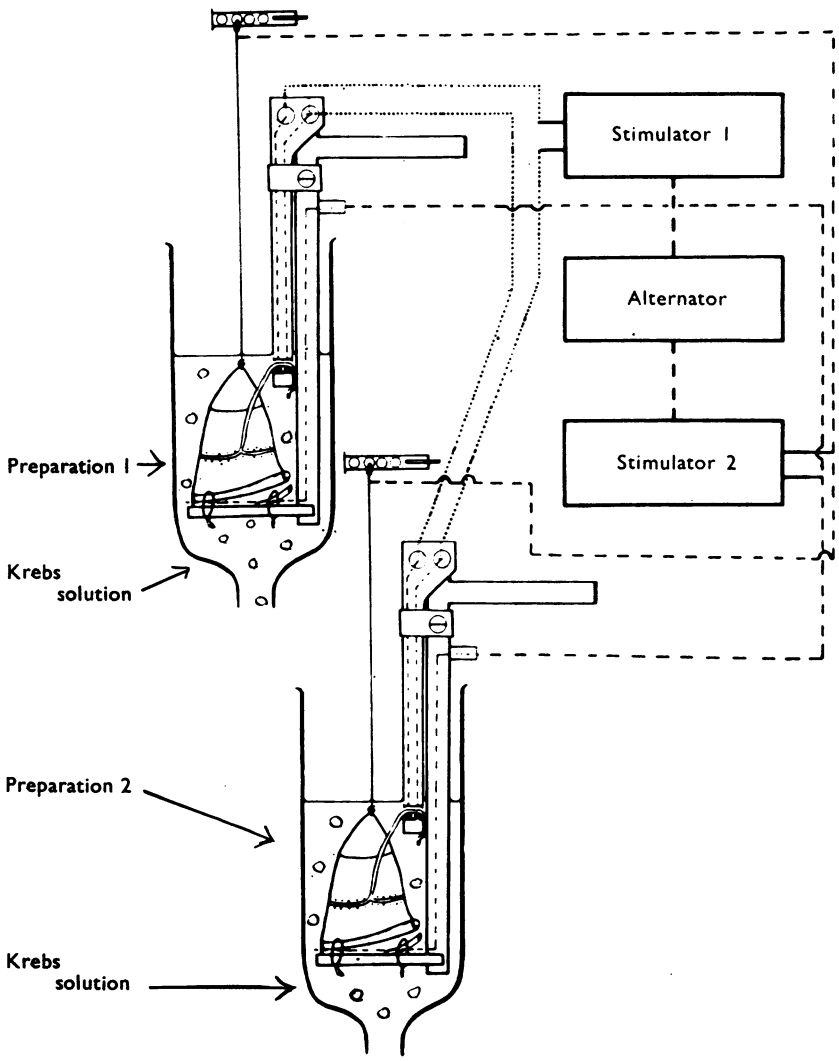

Fig. 1. Diagram of apparatus for stimulating isolated phrenic nerve-diaphragm preparations in pairs.

on a kymograph. Each heart beat caused a single impulse to pass through the circuit to the Thorp counter, whose recording lever rose by 1 mm. After 5 sec the counter was reset automatically to the base-line. Thus multiplication of the height of the trace (in mm) by twelve approximated closely to the heart rate (in beats/min). Occasionally a cathode-ray oscilloscope was substituted for the Thorp counter.

Isolated phrenic nerve-diaphragm preparation. The technique of Bülbring (1946) was modified so that two preparations, one thiamine-deficient and one littermate control, were stimulated supramaximally by rectangular pulses alternately directly to the muscle and indirectly via the nerve at 12 shocks/min (i.e., six indirect and six direct stimuli). The stimulators were similar to those described by Bell (1957). Each diaphragm was set up in a separate 50 ml. organ-bath in Krebs solution at 37° C, bubbled with a mixture of 5% CO_2 and 95% O_2 (Fig. 1). Records from the two preparations were made on a single kymograph paper, one above the other.

The neuromuscular blocking activities of tubocurarine and gallamine were estimated with pairs of diaphragms according to the method of Chou (1947). The action of eserine was studied by measuring the amplitude of the twitch response to single indirect stimuli, before and after addition of the anti-cholinesterase, which was left in the organ-baths for 15 to 20 min. The effects of eserine were also studied with indirect faradic stimulation. First both diaphragms were stimulated supramaximally at 75 shocks/sec for 1, 3, 5 and 10 sec, the magnitude of

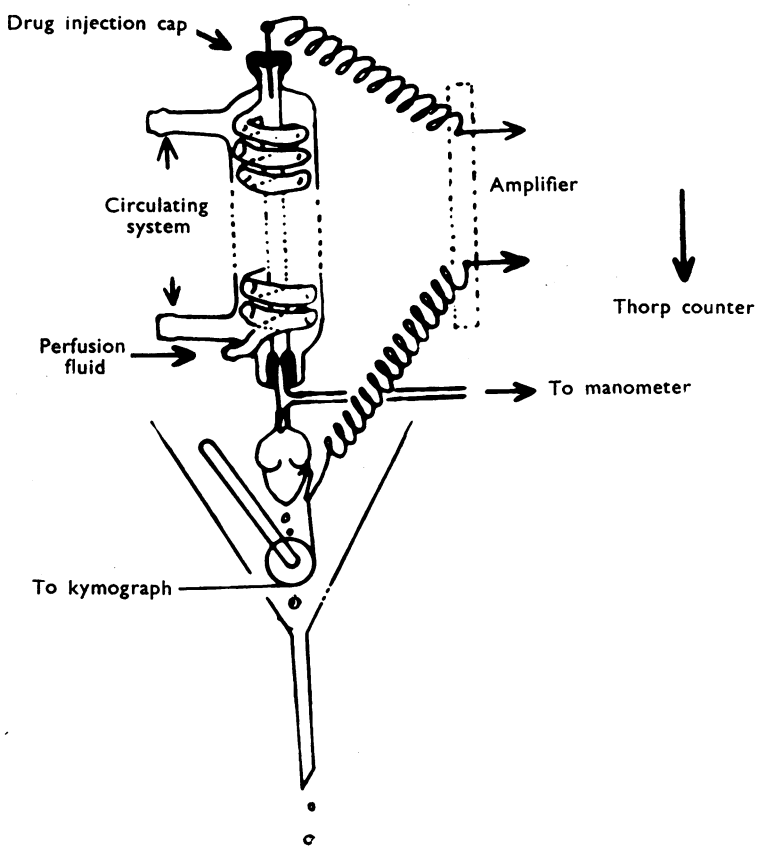

Fig. 2. Diagram of Langendorff apparatus for perfusion of isolated rat heart.

the muscle contractions being measured. Then eserine was added as before, and the faradic stimulation repeated after 5 to 10 min. The decrease in height of contraction was measured and expressed as a percentage of the original response.

Isolated perfused heart preparation. The Langendorff technique was modified by the addition of a water-jacket to surround the heart to maintain an even temperature (Baker, 1951). The heart was perfused with Krebs solution at a pressure of 80 cm H_2O, bubbled with 5% CO_2 and 95% O_2. The heart rate was recorded electrically throughout the experiments, with the apparatus used for recording the rate *in vivo*. One electrode was attached to the right auricle, and the other made contact with the Krebs solution about to enter the heart (Fig. 2). The amplitude of the heart beat was recorded with an isotonic writing lever.

Procedure. Littermate pairs of animals (one thiamine-deficient and one pair-fed control) were killed by decapitation after about 4 weeks on the experimental diets, and appropriate tissues were removed. Experiments with the phrenic nerve-diaphragm preparation were carried out simultaneously as described; the thiamine-deficient isolated heart was studied first and the control on the following day.

Adrenaline acid tartrate B.P., isoprenaline sulphate B.P., (−)-noradrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate and acetylcholine chloride are expressed as weights of the bases. Other drugs are expressed as weights of the salts.

RESULTS

Nutritional aspects have been reported previously (Boullin, 1960, 1961). Severe bradycardia and convulsions usually appeared in the thiamine-deficient animals in the fourth week on the thiamine-deficient diet. The control animals never showed any convulsions, but they were often moribund from inanition, when they occasionally had an extremely low heart rate similar to that previously found by Parade (1938). This only developed on the day before death, and was quite different from the gradually developing bradycardia characteristic of thiamine-deficient rats. The administration of thiamine hydrochloride by mouth always abolished the bradycardia in the deficient rats, but never in the controls. These invariably died unless extra food was given. The responses of pair-fed control rats did not differ from those of rats fed either the complete diet *ad libitum* or diet 41b (Boullin, 1960).

TABLE 1

COMPARATIVE SENSITIVITIES OF THIAMINE-DEFICIENT AND LITTERMATE CONTROL PHRENIC NERVE-DIAPHRAGM PREPARATIONS TO TUBOCURARINE AND GALLAMINE

D, thiamine deficient; C, pair-fed littermate controls. Experiments are listed in order of degree of deficiency determined by "bradycardia test"

	Heart rate (beats/min)		Log ED50, μg			
			Tubocurarine		Gallamine	
Expt.	D	C	D	C	D	C
1	180	470	1·58	1·54	3·84	3·75
2	260	490	1·70	1·76	3·85	3·81
3	290	480	1·64	1·64	3·84	3·81
4	290	505	1·74	1·63		
5	310	480	1·64	1·64	3·78	3·78
6	310	530	1·72	1·62		
7	325	480	1·40	1·55	3·54	3·90
8	360	505	1·73	1·63	3·83	3·72
9	360	480	1·70	1·62	3·82	3·78
Mean			1·65	1·63	3·79	3·79
s.e.±			0·04	0·02	0·04	0·02
P			>0·05		>0·05	

Isolated phrenic nerve-diaphragm preparation

Experiments were made with nine pairs of diaphragm preparations. The neuromuscular blocking activity of tubocurarine and gallamine was estimated from log dose/response curves. The dose of each drug required to produce 50% neuromuscular block (ED50) was estimated. There was no significant difference (at the 5% level) between ED50 values for thiamine-deficient and control preparations with either drug (Table 1).

The action of eserine was studied in six paired experiments. Whereas in control preparations eserine produced a sustained increase of from 32 to 114% of the twitch response to indirect stimulation (Bülbring, 1946), this was not so in thiamine-deficient diaphragms in all six experiments (Table 2). In two of these no potentiation whatsoever was seen with eserine (0.1 to 1.0 μg/ml.), and in the other four

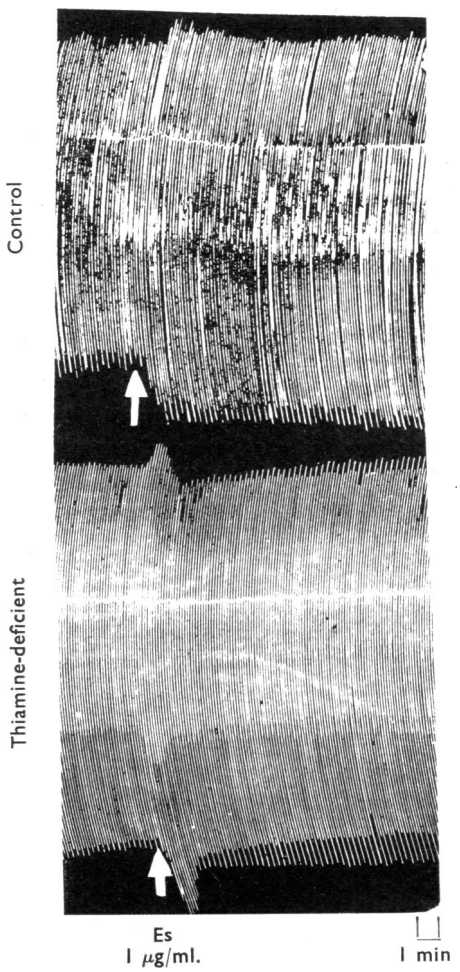

Fig. 3. Abnormal potentiation of twitch response of thiamine-deficient phrenic nerve-diaphragm preparation by eserine (1.0 μg/ml. at arrows). Upper trace, control preparation; lower trace, thiamine-deficient preparation.

experiments the potentiation was quantitatively less, and was also transient (Fig. 3). The difference in the percentage potentiations of thiamine-deficient and control groups was significant at the 1% level.

TABLE 2

POTENTIATING ACTIVITY OF ESERINE ON THIAMINE-DEFICIENT AND LITTERMATE CONTROL PHRENIC NERVE-DIAPHRAGM PREPARATIONS

D, thiamine deficient; C, pair-fed littermate controls; T, transient potentiation, lasting less than 3 min; P, prolonged potentiation, lasting longer than 15 min. Experiments listed in order of degree of deficiency determined by " bradycardia test "

	Heart rate (beats/min)		Maximum potentiation of twitch response (%)		Type of potentiation	
Expt.	D	C	D	C	D	C
1	260	490	0	79	—	P
2	290	505	0	46	—	P
3	325	480	17	114	P	P
4	335	455	18	100	P	P
5	350	480	50	52	T	P
6	350	430	29	32	T	P
Mean			19·0	70·5		
s.e.±			7·7	13·2		
P				<0·01		

For one thiamine-deficient preparation (experiment 6, Table 2) immersion in thiamine pyrophosphate (1 μg/ml.) for 1 hr modified the response to eserine from a transient potentiation of 29% lasting only 2 min to a prolonged potentiation of 12% lasting over 15 min.

In the four experiments where the action of eserine was studied with faradic stimulation, there was no significant difference (at the 5% level) in the percentage reductions of the muscle responses of thiamine-deficient and control groups (Table 3).

TABLE 3

NEUROMUSCULAR BLOCKING ACTIVITY OF ESERINE ON THIAMINE-DEFICIENT AND LITTERMATE CONTROL PHRENIC NERVE-DIAPHRAGM PREPARATIONS WITH FARADIC STIMULATION

D, thiamine deficient; C, pair-fed littermate controls

	Degree of neuromuscular block (%)	
Expt.	D	C
1	97	97
2	96	39
3	97	48
4	58	31
Mean	87·0	53·8
s.e.±	9·7	14·9
P	>0·05	

Isolated perfused heart preparation

Normal hearts. The heart rate of both normal and pair-fed rats *in vivo* was about 500 beats/min (mean 476, s.e.± 16, n=20), but the isolated heart usually beat at 300 to 400 beats/min (mean 336, s.e.± 14, n=20). Although this rapid

rate seemed to indicate a good physiological state of the preparations, the responses to drugs were difficult to reproduce. Without the addition of drugs to the perfusion fluid, the control preparations usually beat for up to 2.5 hr; during this time, although the rate slowed it never declined by more than about 25%. The amplitude of beat and the coronary flow of perfusate declined by over 50%. When drugs were added to the perfusion fluid the survival time was shortened.

The threshold of response to acetylcholine usually lay between 0.01 and 0.1 μg. The response could be conveniently divided into three phases. First, an initial stimulation (phase I); second, inhibition (phase II); and third, secondary stimulation (phase III). Phase I occurred immediately the drug reached the heart and persisted for up

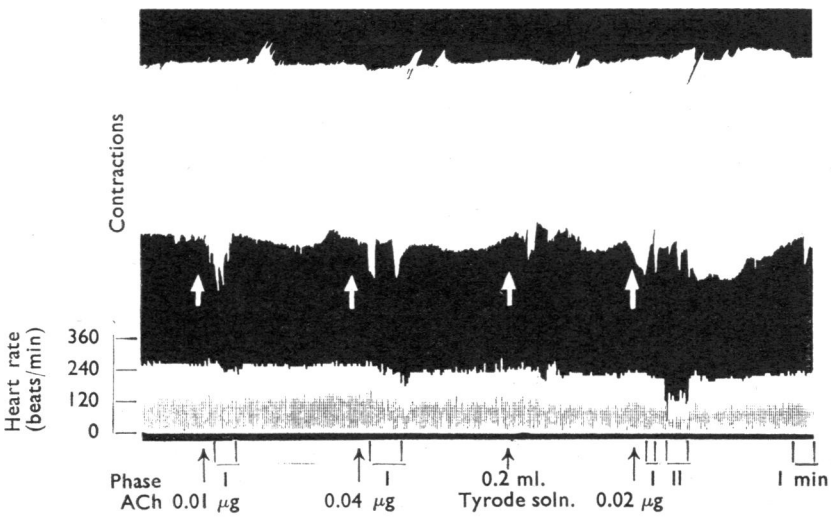

Fig. 4. Effect of acetylcholine (0.01 to 0.4 μg) on control isolated perfused rat heart, showing positive inotropic response (phase I). Upper trace, contractions of heart; lower trace, heart rate.

to 2 min. It consisted of a transient increase in amplitude of beat unaccompanied by any pronounced change in heart rate (Fig. 4), and was normally only seen with small doses of acetylcholine.

The inhibition (phase II) occurred so regularly that it might be termed the "typical" response of the rat heart to acetylcholine. The most prominent feature of this phase was bradycardia leading sometimes to cardiac arrest (Fig. 5). The accompanying inotropic changes were variable, and seemed to be related to the heart rate; the greatest negative chronotropic effects were associated with the greatest negative inotropic effects. Vane (1957) has shown that an interaction of chronotropic and inotropic effects may occur under certain circumstances.

The secondary stimulation (phase III) was only seen with large doses of acetylcholine (10 μg or more), and was characterized by an increase in amplitude and rate of beat, mimicking the effects of adrenaline (Fig. 6). Phase III often did not appear until several minutes after the administration of acetylcholine, but persisted for up to 10 min.

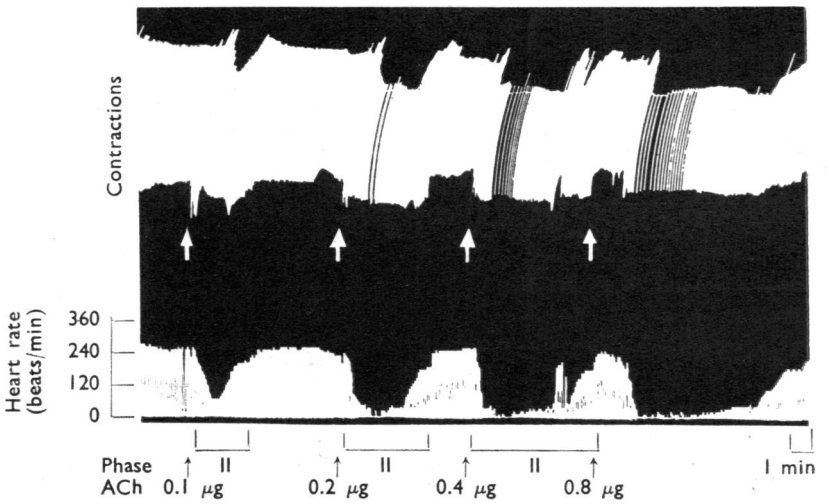

Fig. 5. Effect of acetylcholine (0.1 to 0.8 μg) on normal isolated perfused rat heart, showing negative chronotropic and inotropic responses (phase II). Upper trace, contractions of heart; lower trace, heart rate.

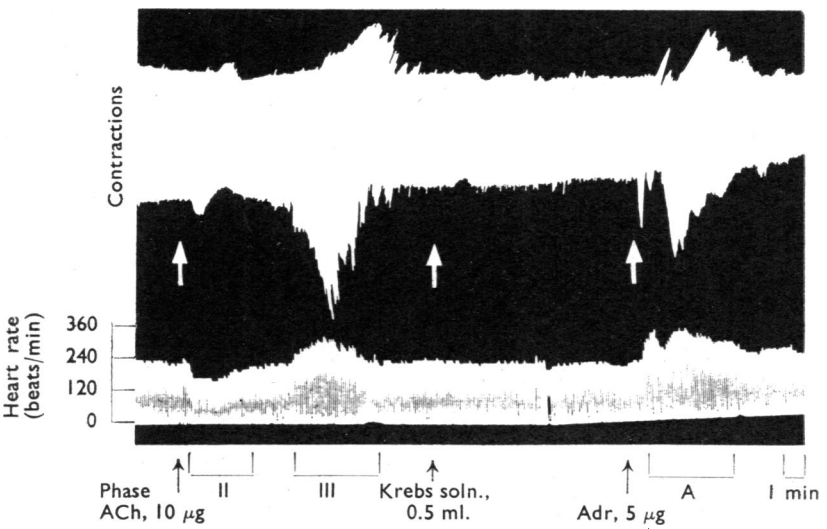

Fig. 6. Similarity of actions of acetylcholine (10 μg) and adrenaline (5 μg) on control isolated perfused rat heart. Upper trace, contractions of heart; lower trace, heart rate.

The actions of adrenaline, noradrenaline and isoprenaline were qualitatively similar (Fig. 7), and were also divisible into three phases. Phase A, pronounced stimulation, consisted of a positive chronotropic response usually accompanied by a spectacular increase in amplitude of beat (up to 200%). This was followed by phase B, a period of slowing and irregularity of heart rate sometimes with heart block; the atria usually continued beating, as observed directly and with a cathode ray oscilloscope. Both phase A and phase B were influenced by the prevailing heart rate. When the initial heart rate was low the phase A response was large

(Fig. 7), and phase B was virtually absent. Conversely, when the initial rate was high, the phase A response was diminished or absent and phase B was prominent (Fig. 8). The third phase, C, was a continuation of the initial stimulation (phase A) and was indistinguishable from it unless phase B intervened.

As the responses of the isolated heart of the rat were so variable, it was not possible to construct reliable log dose/response curves. However, isoprenaline appeared consistently to be ten times more potent than either adrenaline or nor-

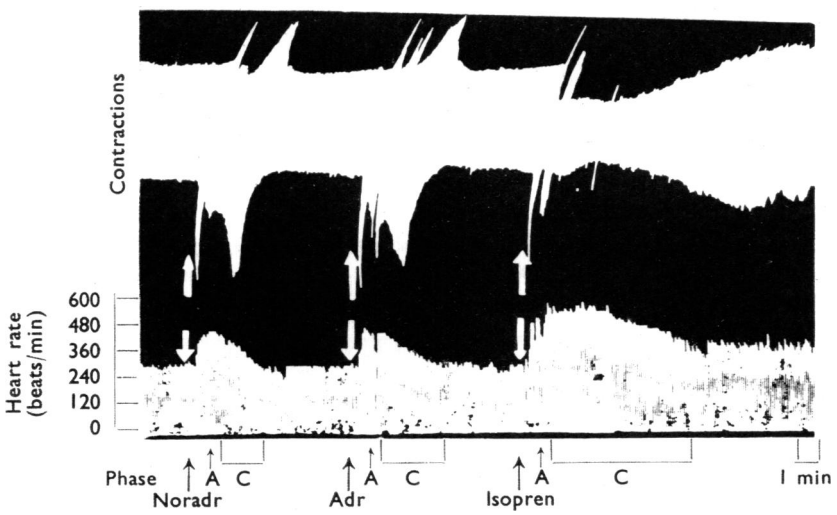

Fig. 7. Stimulation of the control isolated perfused rat heart by equal doses (0.1 μg) of sympathomimetic amines, showing only phase A and C responses. Upper trace, contractions of heart; lower trace, heart rate.

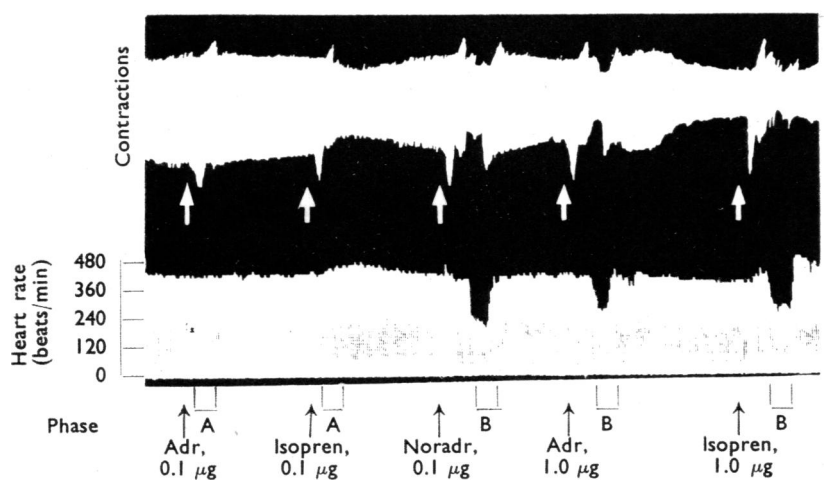

Fig. 8. Effect of sympathomimetic amines on control isolated perfused rat heart, showing prominence of phase B responses with the higher doses (1.0 μg). Upper trace, contractions of heart; lower trace, heart rate.

adrenaline (which were equipotent), on the basis of the doses required for equivalent positive chronotropic responses.

Thiamine-deficient hearts. The heart rate *in vivo* was never more than 360 beats/min (mean 311, s.e. ± 12, n = 20). *In vitro* the heart rate was unchanged in six out of twenty experiments (Table 4), and in two experiments (animals with severe bradycardia) there was a rise in the rate. However, the mean figures showed a significant reduction (at the 1% level) for the heart rates of thiamine-deficient rats.

TABLE 4

THE ACTION OF ADRENALINE ON THIAMINE-DEFICIENT AND LITTERMATE CONTROL ISOLATED PERFUSED HEART PREPARATIONS

D, thiamine deficient; C, pair-fed littermate controls. Experiments listed in order of degree of deficiency determined by "bradycardia test." * D>C=+; D<C=−

Expt.	Heart rates D/C (beats/min) *In vivo*	Heart rates D/C (beats/min) *In vitro*	Sensitivity difference*	Cardiac arrest D	Cardiac arrest C	Increase in heart rate, *in vitro : in vivo* D	Increase in heart rate, *in vitro : in vivo* C	Phase A, inotropic effect D	Phase A, inotropic effect C
1	180/265	288/205	Nil			+			
2	190/480	180/190	+10	+					+
3	215/540	265/370	−10	+		+			+
4	265/505	265/370	Nil			+		+	+
5	300/300	300/300	Nil			+	+		+
6	300/480	275/370	−4	+				+	+
7	310/505	230/350	+10	+				+	+
8	310/470	275/350	−10	+	+				+
9	325/490	290/360	+8						+
10	325/515	290/395	+5						+
11	325/530	325/385	+100	+		+			+
12	335/540	335/420	+8	+		+		+	+
13	335/480	275/335	+5						+
14	350/420	265/310	+10	+	+				+
15	350/505	300/410	+4	+	+			+	+
16	360/505	310/350	Nil	+					+
17	360/445	180/265	+8	+					+
18	360/530	325/310	Nil						+
19	360/515	360/325	+15	+		+			+
20	360/490	360/350	Nil	+		+		+	+
Mean	311/476	285/336							
s.e.±	12/16	11/14							
P	<0·001	<0·01							

The responses to acetylcholine of nine preparations did not differ qualitatively from those seen in littermate controls. Nevertheless, the thiamine-deficient hearts had increased sensitivity to acetylcholine in that the threshold of response was lower, and the heart was arrested with smaller doses. In seven out of nine experiments the dose required for cardiac arrest was 1/2 to 1/40 of that for the littermate controls (Table 5).

Adrenaline, noradrenaline and isoprenaline were studied in twenty paired experiments (Table 4). The thiamine-deficient preparations showed some or all of the three stages of response seen with normal hearts, with certain qualitative and quantitative differences. First, phase A was absent in fourteen experiments. Second, phase B was more prominent and the irregularities were progressive, often leading to cardiac arrest; this was seldom seen in control experiments.

TABLE 5

SENSITIVITY DIFFERENCES OF THIAMINE-DEFICIENT AND LITTERMATE CONTROL ISOLATED HEART PREPARATIONS TO ACETYLCHOLINE

D, thiamine deficient; C, pair-fed littermate controls. Experiments listed in order of degree of deficiency determined by "bradycardia test." * D>C=+; D<C=−

Expt.	Heart rates D/C (beats/min) *In vivo*	*In vitro*	Sensitivity difference*
1	265/505	265/370	+10
2	310/505	230/250	+4
3	325/515	290/395	−2
4	325/530	325/385	+2
5	335/480	275/335	+8
6	360/505	310/350	+8
7	360/530	325/310	+40
8	360/515	360/325	+40
9	360/490	360/350	Nil
Mean	333/508	304/341	
s.e.±	11/6	15/15	
P	<0·001	>0·05	

Thiamine-deficient hearts had increased sensitivity to adrenaline, noradrenaline and isoprenaline, computed on the basis of the doses required to produce equivalent positive chronotropic effects. Of twenty deficient hearts, eleven were 4 to 100 times more sensitive, six were equally sensitive, and three were less sensitive than the controls.

DISCUSSION

It is generally assumed that the defects of thiamine deficiency are limited to "biochemical lesions" (Gavrilescu & Peters, 1931; Peters, 1936) associated with several aspects of intermediary metabolism (Fruton & Simmonds, 1958), including the conversion of pyruvate to acetyl co-enzyme A. As this co-enzyme is the ultimate source of the acetyl group of acetylcholine (Birks & MacIntosh, 1957), on theoretical grounds it is likely that acetylcholine synthesis is impaired in thiamine deficiency. There is evidence that this is so (Mann & Quastel, 1940; Bhagat & Lockett, 1962a), and brain acetylcholine levels are reduced in thiamine-deficient rats (Lissak, Kovacs & Nagy, 1943). Nevertheless, the defects do not seem to extend to the skeletal neuromuscular junction, since the responses to tubocurarine and gallamine of thiamine-deficient diaphragm preparations were normal and the potency ratio (tubocurarine : gallamine) of 100 : 1 was unaltered by deficiency. This value is close to that of 80 : 1 reported by Wien (1948), while the ED50 for gallamine found by Bülbring & Depierre (1949), namely 6 to 8 mg, agrees well with the value found here (mean 6.17 mg, s.e. ± 0.042; n = 7).

The results reported here do not agree with the findings of Bhagat & Lockett (1962b) that thiamine-deficient diaphragms showed subnormal twitch tensions in response to single indirect stimuli via the nerve, and subnormal responses to both direct and indirect faradic stimulations. However, as they compared preparations from thiamine-deficient with those from normal animals, they did not eliminate differences in body weight due to the inanition that accompanies thiamine deficiency (Reader & Drummond, 1926).

Few studies appear to have been made of the pharmacological responses of thiamine-deficient tissues. Martin & Lissák (1941) found no difference in the response of the isolated, thiamine-deficient rat heart to acetylcholine, but others have reported increased sensitivity of the thiamine-deficient intestine (Beauvallet, 1938 ; Andra & Blaizot, 1956).

Other workers have noted pharmacological effects of high concentrations of thiamine on the phrenic nerve-diaphragm preparation (Cheymol, Bourillet, Levassort & Kerp, 1957) and on frog skeletal muscle (Torda & Wolff, 1944). However, the active form of thiamine is thiamine pyrophosphate (Peters, 1953) and responses to high concentrations of thiamine are not strictly physiological.

One explanation of the differences reported here between thiamine-deficient and control tissues is that the biochemical defects associated with deficiency limit the supplies of energy available to the isolated tissues (Bhagat & Lockett, 1962a). This limitation might be expected to impair the ability of tissues to respond to those drugs, such as sympathomimetic amines, which normally produce stimulation, and this explanation would account for such qualitative differences as the absence of phase A and prominence of phase B effects. It would also account for the failure of eserine to produce sustained potentiation of the twitch response of the thiamine-deficient diaphragm, and the remedial effect of thiamine pyrophosphate in one experiment supports this explanation.

The comparatively small quantitative differences in these experiments serve to emphasize that the defects of thiamine deficiency are probably limited to biochemical lesions. The bradycardia in thiamine-deficient rats may be neurogenic (Carter & Drury, 1929), though Beznak (1956) found that the bradycardia was not abolished by vagotomy. However, the most plausible explanation is that the bradycardia is due to inhibition of the tricarboxylic acid cycle. Not only have Peters & Wakelin (1953) shown that injection of fluorocitrate, an inhibitor of the cycle, into the subarachnoid space of pigeons produced signs closely resembling those of thiamine deficiency, but Peters (1953) found that this compound in rats caused a bradycardia mimicking that of thiamine deficiency.

One interesting observation was the stimulant effect of acetylcholine on the normal rat heart. Similar observations have been made with the rabbit, cat and dog (McDowall, 1946a and b ; Hoffmann, Hoffmann, Middleton & Talesnik, 1945). The latter workers detected a substance like adrenaline in the perfusate when acetylcholine was applied, and suggested that this substance released a sympathomimetic agent which they believed was derived from either cardiac chromaffin tissue or sympathetic ganglia. The view of Burn (1961), that stimulation of all postganglionic sympathetic neurones liberates acetylcholine which in turn releases noradrenaline, is supported by the close similarity between the actions of certain doses of acetylcholine and those of the sympathomimetic amines which were examined with the rat heart.

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REFERENCES

ANDRA, A. M. & BLAIZOT, J. (1956). Carance en aneurine et motricité intestinale. *C.R. Soc. Biol. (Paris)*, **150**, 1713–1721.

BAKER, J. B. E. (1951). An improved apparatus for mammalian heart perfusion. *J. Physiol. (Lond.)*, **115**, 30–32*P*.

BEAUVALLET, M. (1938). Sensibilité à l'acetylcholine de l'intestin grêle isolé du pigeon normal et du pigeon atteint de polynévrité. *C.R. Soc. Biol. (Paris)*, **128**, 1020–1021.

BELL, P. M. G. (1957). Stimulator control to provide single shocks alternately to nerve and muscle with faradic stimulation of the nerve at predetermined intervals. *J. Physiol. (Lond.)*, **137**, 1–2*P*.

BEZNAK, A. B. L. (1956). The effect of thiamine deficiency, food restriction, vagotomy and thiamine injection on the heart rate in rats. *Int. Z. Vitamin-forsch.*, **27**, 153–168.

BHAGAT, B. & LOCKETT, M. F. (1962a). The synthesis of acetylcholine by acetone dried powders from the brains of normal rats and of thiamine-deficient rats. *J. Pharm. Pharmacol.*, **14**, 37–40.

BHAGAT, B. & LOCKETT, M. F. (1962b). The effect of deficiency and small excess of thiamine on the rat phrenic nerve diaphragm preparation. *J. Pharm. Pharmacol.*, **14**, 161–168.

BIRKS, R. I. & MACINTOSH, F. C. (1957). Acetylcholine metabolism at nerve endings. *Brit. med. Bull.*, **13**, 157–161.

BOULLIN, D. J. (1960). Pharmacological responses of vitamin B_1 deficient rat tissues. M.Sc. Thesis, London University.

BOULLIN, D. J. (1961). Thiamine requirement of rats given a high-protein, carbohydrate-free diet. *Brit. J. Nutr.*, **15**, 577–586.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation. *Brit. J. Pharmacol.*, **1**, 38–61.

BÜLBRING, E. & DEPIERRE, F. (1949). The action of synthetic curarising compounds on skeletal muscle and sympathetic ganglia both normal and denervated. *Brit. J. Pharmacol.*, **4**, 22–28.

BURN, J. H. (1961). A new view of adrenergic nerve fibres explaining the action of reserpine, bretylium and guanethidine. *Brit. med. J.*, **i**, 1623–1627.

CARTER, R. C. W. & DRURY, A. N. (1929). Heart block in rice fed pigeons. *J. Physiol. (Lond.)*, **68**, 1–2*P*.

CHEYMOL, J., BOURILLET, F., LEVASSORT, C. & KERP, L. (1957). Sur les actions curarisantes et anticurares de la thiamine et des dérivés. *Arch. int. Pharmacodyn.*, **111**, 36–59.

CHOU, T. C. (1947). A method of estimating curare-like activity on the isolated phrenic nerve diaphragm preparation of the rat. *Brit. J. Pharmacol.*, **2**, 1–7.

DRURY, A. N., HARRIS, L. J. & MAUDSLEY, C. (1930). Vitamin B_1 deficiency in the rat. Bradycardia as a distinctive feature. *Biochem. J.*, **24**, 1632–1649.

FRUTON, J. S. & SIMMONDS, S. (1958). *General Biochemistry*, 2nd ed., pp. 475–483, 504–505, 529–531. New York: Wiley.

GAVRILESCU, N. & PETERS, R. A. (1931). On the function of Torulin. An *in vitro* effect of anti-neuritic vitamin concentrates. *Biochem. J.*, **25**, 2150–2161.

HOFFMANN, F., HOFFMANN, E. J., MIDDLETON, S. & TALESNIK, J. (1945). The stimulatory effect of acetylcholine on the mammalian heart, and the liberation of an epinephrine-like substance by the isolated heart. *Amer. J. Physiol.*, **144**, 189–198.

KON, S. K. & DRUMMOND, J. C. (1927). The physiological role of vitamin B_1. Part III, study of vitamin B deficiency in pigeons. *Biochem. J.*, **21**, 632–652.

LISSAK, K., KOVACS, T. & NAGY, E. K. (1943). Acetylcholin- und cholinesterasegehalt von organen B_1-avitaminotischer und normaler ratten. *Pflügers Arch. ges. Physiol.*, **247**, 124–131.

MCDOWALL, R. J. S. (1946a). The stimulatory action of acetylcholine on the heart. *J. Physiol., (Lond.)*, **104**, 41*P*.

MCDOWALL, R. J. S. (1946b). The production of an adrenaline-like substance by the heart. *J. Physiol. (Lond.)*, **104**, 392–403.

MANN, P. J. G. & QUASTEL, J. H. (1940). Vitamin B_1 and acetylcholine formation in isolated brain. *Nature (Lond.)*, **145**, 856–857.

MARTIN, J. & LISSÁK, K. (1941). Azetylcholinempfindlichkeit und vitamin B_1. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **198**, 667–674.

MURALT, A. VON (1947). Thiamine and peripheral neurophysiology. *Vitam. and Horm.*, **5**, 93–118.

NABER, E. C., CRAVENS, W. W., BAUMANN, C. A. & BIRD, H. R. (1954). The effect of thiamine analogs on embryonic development and growth of the chick. *J. Nutr.*, **54**, 579–591.

PARADE, G. W. (1938). Vitamin B-untersuchungen. *Z. vitaminforsch.*, **7**, 35–45.

PETERS, R. A. (1936). The biochemical lesions in vitamin B_1 deficiency. *Lancet*, **i**, 1161–1164.

PETERS, R. A. (1953). Significance of biochemical lesions in the pyruvate oxidase system. *Brit. med. Bull.*, **9**, 116–121.

PETERS, R. A. & WAKELIN, R. W. (1953). Pyruvate oxidase system in brain tissue. *J. Physiol. (Lond.)*, **119**, 421–427.

READER, V. & DRUMMOND, J. C. (1926). Relation between vitamin B and protein in the diet of growing rats. Physiological role of vitamin B. *Biochem. J.*, **20**, 1256–1263.

SANDELS, M. R. (1930). Experimental nutritional polyneuritis in the rat. *J. Nutr.*, **2**, 409–413.

TORDA, C. & WOLFF, H. C. (1944). Effect of vitamin B_1 and co-carboxylase on synthesis of acetylcholine. *Proc. Soc. exp. Biol. (N.Y.)*, **56**, 88–89.

VANE, J. R. (1957). Frequency of contraction as a factor in the action of drugs on isolated heart tissue. *J. Physiol. (Lond.)*, **138**, 16–17*P*.

WIEN, R. (1948). The curarising properties of R.P. 3697. *Arch. int. Pharmacodyn.*, **77**, 96–99.

WOOLLEY, D. W. (1951). An enzymatic study of the mode of action of pyrithiamine. *J. biol. Chem.*, **191**, 43–54.

WOOLLEY, D. W. & MERRIFIELD, R. B. (1954). Mise en évidence d'une nouvelle action de la thiamine par l'emploi de la pyrithiamine. *Bull. Soc. Chim. biol. (Paris)*, **36**, 1207–1212.