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DISTURBANCE OF OXIDATIVE PHOSPHORYLATION BY AN ANTIBIOTICUM PRODUCED BY PSEUDOMONAS COCOVENENANS

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Abstract—The effect of a very toxic agent with antibiotic properties (bongkrekic acid), produced by *Pseudomonas cocovenenans*, on the oxidation of citric acid cycle intermediates catalyzed by heart muscle tissue has been investigated. It was found that the oxidation of pyruvate, α-ketoglutarate and malate was inhibited, whereas oxidation of succinate and β-hydroxybutyrate was stimulated. The phosphorylation, coupled with these oxidations proved to be even more sensitive to bongkrekic acid. The concentration required to produce these effects on isolated mitochondria is very low; in most cases 10^{-6} M is very effective.

SEVERAL years ago Van Veen and Mertens[1, 2] discovered that large-scale food intoxications on Mid-Java (Indonesia) were caused by a hitherto unknown micro-organism, *Pseudomonas cocovenenans*, growing on "bongkrek", a product prepared by the natives from defatted coconut. The main toxic agent produced by this bacterium turned out to be an acid and was called bongkrekic acid. This substance could be extracted from cultures of *Pseudomonas cocovenenans* on defatted copra by the same investigators.[3]

The chemical structure of bongkrekic acid has been partially elucidated by Nugteren and Berends.[4] The bruto formula is $C_{29}H_{40}O_7$ and the substance was shown to be branched olefinic tricarboxylic acid with a tertiary methoxyl group in a δ-position with respect to one of the carboxylic acid groups. Seven double bonds are present forming at least two conjugated systems. Hydrogenated bongkrekic acid is no longer toxic. In acid solutions the unsaturated compound is very unstable and decomposes rapidly to non-toxic products.

The pharmacological properties of bongkrekic acid have been studied by van Veen and Mertens[5] and recently also by von Holt.[6] The toxicity was tested on several species (monkeys, rabbits, rats and pigeons) and the product found to be lethal in every instance. Administration of 2 mg/100 g body weight to rats caused death within 2–5 hr; 1 mg/100 g was tolerated, but the same dose when repeated after 48 hr proved lethal.

Oral administration of bongkrekic acid initially produces hyperglycaemia, soon followed by a severe hypoglycaemia. Intravenous injections of glucose give some relief but do not prevent death. It could be shown that just before death all glycogen had disappeared from liver and heart. The lactic acid content of the blood increases to two to three times the normal value.

Finally it was found[7] that bongkrekic acid also possesses antibiotic properties towards many micro-organisms (yeasts, bacteria and molds).

Based on these facts an investigation was set up to get some insight into the mode

of action of this remarkable substance. After the failure of some glycolytic enzymes (hexokinase, aldolase, isomerase, 3-phosphoglyceraldehyde dehydrogenase) to show any sensitivity to bongkrekic acid, attention was directed to the citric acid cycle. In preliminary experiments we used a homogenate of sheep heart muscle; later rat heart mitochondria were used as enzyme source. Finally the investigation was extended to include a soluble α-ketoglutaric acid oxidase preparation.

MATERIALS AND METHODS

Sheep heart was obtained at a local slaughterhouse; it was removed from the body about 15 min after death of the animal and chopped into pieces of a few g. It was transported to the laboratory cooled with solid carbon dioxide and stored at −25°C for some months without appreciable loss of activity. Homogenates were prepared with a Bühler high-speed blendor. One or two lumps of frozen tissue were thawed and blendored in the phosphate buffer described by Krebs and Eggleston[8] for 45 sec; the blendor vessel was cooled with melting ice.

Rat heart mitochondria were isolated as described by Cleland and Slater[9] in 0·25 M sucrose – 0·01 M versene, pH 7·4, and suspended in 0·25 M sucrose (neutralized to phenol red).

Oxygen uptake was followed in a Warburg apparatus (air as gas phase) at 37 or 30 °C. Inorganic ortho-phosphate was assayed by the FiskeSubbaRow method. Oxidative phosphorylation was measured by the disappearance of inorganic phosphate from the incubation medium with glucose plus hexokinase (prepared according to Berger *et al.*[10] and purified until step 4) as the trapping system for ATP.* One of two identically filled Warburg vessels was deproteinized by tipping in 0·5 ml 17·5% trichloroacetic acid from the side arm after an equilibration period of 10 min in the waterbath; this vessel was used to assay the inorganic phosphate concentration at zero time. With the other vessel oxygen uptake was measured until the reaction was stopped in the same manner; inorganic phosphate was measured in the deproteinized fluid.

Samples of bongkrekic acid were generously supplied by Dr. D. H. Nugteren (for preparation see [4]) as solutions in 2·5% bicarbonate. These preparations contained about 25 % saturated fatty acids with 6–10 carbon atoms, chiefly capronic acid. The concentration of the samples was polarimetrically or spectrophotometrically determined. For removal of bicarbonate the BA-solutions were acidified with 2 N sulphuric acid and, immediately after the formation of a white flocculent precipitate, extracted with peroxide-free ether. After a washing of the ethereal solution with water BA was extracted from it with small portions of a 2·5% solution of potassium phosphate. Residual ether was removed under vacuum until its smell no longer could be detected.

Cytochrome-*c* was prepared as described by Keilin and Hartree[11]; the last purification step consisted in column chromatography on Amberlite IRC-50 according to Margoliash.[12]

α-Ketoglutaric acid dehydrogenase and succinate activating enzyme (P-enzyme) were isolated, purified and tested for activity with the methods of Kaufman.[13] α-KG dehydrogenase contained 0·58 mg protein/ml and had a specific activity of 0·42 (specific

* The following abbreviations are used: ADP, ATP: adenosinedi- and tri-phosphate; GDP, GTP: guanosinedi- and tri-phosphate; IDP, ITP: inosinedi- and tri-phosphate; CoA: coenzyme A; DPN: diphosphopyridine dinucleotide; BOH: β-hydroxybutyric acid; DNP: 2:4-dinitrophenol; BA: bongkrekic acid; α-KG: α-ketoglutaric acid; P_i: inorganic ortho-phosphate.

activity is expressed as μmoles α-KG oxidized per min/mg protein). P-enzyme contained 2·9 mg protein/ml and had a specific activity of 0·54 (specific activity expressed as μmoles succinate activated per 30 min/mg protein at 37 °C).

The nitrogen content of the mitochondria suspensions was measured with a Kjeldahl method on semi-micro scale, using the apparatus described by Markham.[14]

RESULTS

1. *Preliminary experiments with sheep heart muscle homogenate*

The homogenates prepared from sheep heart muscle showed a considerable oxygen uptake in the absence of added substrate, most probably due to the oxidation of endogenous substrates such as glycogen.

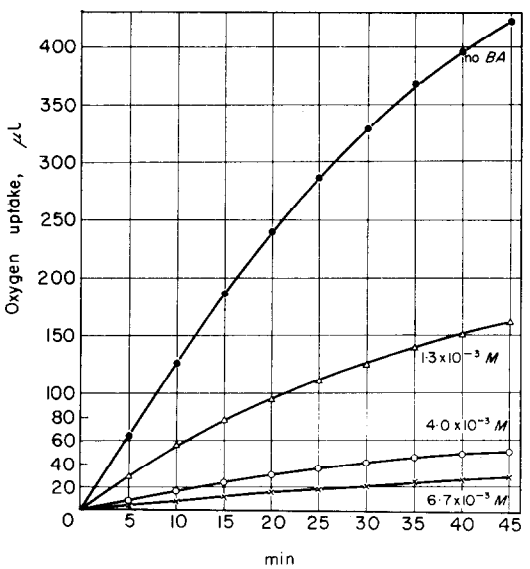

FIG. 1. Inhibition of oxidation of endogenous substrate by BA. A 12% heart muscle homogenate, reinforced with DPN^+ ($4·5 \times 10^{-4}$ M) and buffered at pH 7·4 was incubated at 37 °C with three concentrations of BA.

As may be seen from Fig. 1, this oxygen consumption is inhibited by BA and the inhibition depends on the concentration of the inhibitor.

When the oxygen uptake caused by the oxidation of endogenous substrate was inhibited by the addition of iodoacetic acid to about the same degree as with 0·003 M BA it appeared that the effects of these two inhibitors, when added together, were partially additive. This is shown in Table 1. From studies by Holzer *et al.*[15] it is known that the concentration of iodoacetic acid used will practically inhibit no enzyme of the glycolytic chain other than 3-phosphoglyceraldehyde dehydrogenase and as this enzyme is not sensitive to BA (unpublished experiments) it seemed profitable as a first approach to look for the point of attack of BA among those enzymes of carbohydrate metabolism operative beyond the triosephosphate stage.

Experiments designed to test the sensitivity of the citric acid cycle enzymes to BA were not very successful in consequence of the disturbing effect of the high endogenous oxygen consumption. Addition of citric or α-ketoglutaric acid did not affect the oxygen

TABLE 1. COMPARISON OF THE INHIBITORY EFFECT OF IODOACETIC ACID AND BONGKREKIC ACID ON THE OXYGEN CONSUMPTION OF HEART MUSCLE HOMOGENATE

Additions	Oxygen uptake (μl/45 min)	Inhibition (%)
Experiment 1		
none	198	0
iodoacetic acid $1{\cdot}6 \times 10^{-2}$ M	53	73
bongkrekic acid 3×10^{-3} M	53·5	73
iodoacetic acid + bongkrekic acid	24·5	87·5
Experiment 2		
none	165·5	0
iodoacetic acid $1{\cdot}4 \times 10^{-2}$ M	62·5	62
bongkrekic acid 3×10^{-3} M	55·5	66·5
iodoacetic acid + bongkrekic acid	27·5	83·5

Homogenate, 10% (exp. 1), 7% (exp. 2); DPN, $4{\cdot}5 \times 10^{-4}$ M; cytochrome *c*, 1 mg/ml.

uptake, whereas pyruvic acid reduced oxygen uptake considerably, this reduction being enhanced by BA. The addition of CoA however gave remarkable results. As is shown in Table 2 we found that in the presence of CoA BA had a less inhibitory effect on the O_2-uptake. CoA used in these experiments was a commercial preparation obtained from the Pabst-brewery (Milwaukee, Wisconsin, U.S.A.); it was about 75 % pure. The depression of the O_2-uptake, when large amounts of CoA (600 γ/ml) were added (experiment 3, Table 2) may presumably be ascribed to toxic impurities in this CoA sample.

TABLE 2. CoA INDUCED REVERSAL OF THE INHIBITORY EFFECT OF BONGKREKIC ACID ON THE OXYGEN CONSUMPTION OF HEART MUSCLE HOMOGENATE

Additions	O_2-uptake (μl/45 min)	Inhibition (%)
1. None	462·5	
BA: 3×10^{-3} M	85·5	81
CoA: 200 γ/ml	487·5	
BA + CoA	151	69
2. None	351	
BA: 3×10^{-3} M	63·5	82
CoA: 200 γ/ml	406	
BA + CoA	139·5	66
3. None	351	
BA: 3×10^{-3} M	63·5	82
CoA: 600 γ/ml	192	
BA + CoA (600 γ/ml)	103·5	46

Homogenate, 11% (exp. 1) and 8% (exp. 2 and 3); DPN, 6×10^{-4} M; cytochrome *c*, 1 mg/ml.

In the presence of CoA (200 γ/ml) the addition of citrate gave an extra O_2-uptake; this extra O_2-consumption was almost totally reduced by the addition of BA (Table 3). The oxidation of α-KG seemed not to be very sensitive to BA: the addition of α-KG even had some relieving effect, for the percentage inhibition dropped to about half that of the control at the highest concentration of α-KG used (Table 3). Further experiments however showed that this relieving effect of α-KG did not indicate an

insensitivity of the α-KG oxidase to BA, but that it was most probably caused by a small amount of succinate in the α-KG preparation, because in the presence of malonic acid the relieving effect of α-KG addition was completely absent (Table 3).

TABLE 3. THE EFFECT OF BONGKREKIC ACID ON THE OXIDATION OF CITRATE AND α-KG BY SHEEP HEART HOMOGENATE IN THE PRESENCE OF CoA

Additions	O_2-uptake (μl/hr)		Inhibition (%)
	No BA	BA present	
Exp. 1			
None	588	167	72
citrate	743	199	73
α-KG	653	305	53
Exp. 2			
None	473	146	69
α-KG	619	373	40
malonic acid	99	37	63
α-KG + malonic acid	323	108	66

All Warburg flasks contained $4{\cdot}5 \times 10^{-4}$ M DPN^+ and 200 γ/ml CoA. Further additions: citrate, $5{\cdot}6 \times 10^{-3}$ M; α-KG, $2{\cdot}4 \times 10^{-3}$ M (exp. 1) and $4{\cdot}4 \times 10^{-3}$ M (exp. 2); malonate, 5×10^{-3} M; BA, 3×10^{-3} M. In exp. 1 the concentration of the homogenate was 7·7% and in exp. 2, 8·4%; all measurements were made at 30 °C.

The oxidation of succinic acid however very clearly proved not to be impaired by BA (Fig. 2); oxygen uptake was considerably increased by the addition of succinate and an inhibition of only 10 % was found when BA was present.

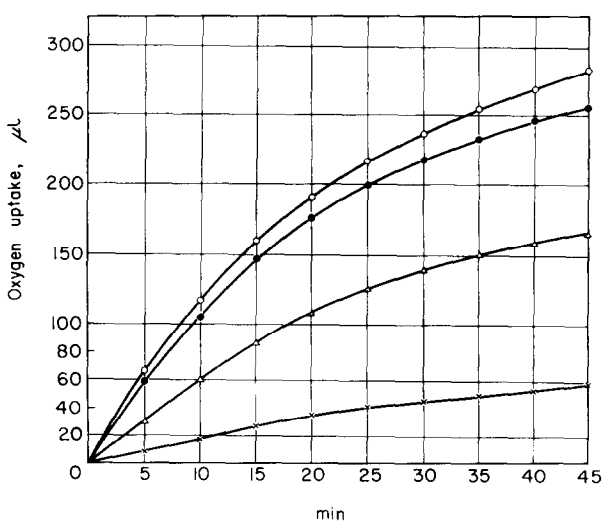

FIG. 2. Decrease of inhibition of oxygen uptake in the presence of succinate. To a heart muscle homogenate (7 per cent) reinforced with $4{\cdot}5 \times 10^{-4}$ M DPN^+ were added BA ($2{\cdot}5 \times 10^{-3}$ M) and succinate ($1{\cdot}2 \times 10^{-2}$ M). △-△-△-△, no addition; ○-○-○-○, succinate; ×-×-×-×, BA; ●-●-●-●, succinate + BA.

2. *Experiments with isolated rat heart muscle mitochondria*

The incubation medium for measuring O_2-uptake and oxidative phosphorylation

was based on that described by Slater and Cleland[16] and Slater and Holton.[17] Some minor modifications (halving of the fluoride and doubling of the Mg^{2+}-concentration) were found to be necessary to obtain optimum P/O-ratios in our hands. With α-KG P/O-ratios between 2·4 and 3·5 (mean 2·7 in 14 experiments) were obtained, and this value was practically independent of the incubation time between 10 and 40 min.

The effect of BA on the oxidative phosphorylation with α-KG as the substrate is shown in Table 4. In this table the results of 4 experiments covering an inhibitor range from 10^{-3} M to 10^{-6} M are summarized. The general conclusion from these results is that both oxygen uptake and phosphorylation are inhibited, but that phosphorylation is more sensitive to BA than oxidation. With increasing concentrations of the inhibitor rather curious results were obtained regarding its effect on the oxygen consumption:

(a) in contrast to what was to be expected the inhibition decreases rather than increases;
(b) the percentage of inhibition never reaches a value of 100 %
(c) the percentage of inhibition varies considerably from experiment to experiment and seems to be related to the amount of mitochondria (the higher the amount of mitochondria the smaller the inhibition).

TABLE 4. THE EFFECT OF BONGKREKIC ACID ON THE OXIDATIVE PHOSPHORYLATION IN RAT HEART MITOCHONDRIA WITH α-KETOGLUTARIC ACID AS SUBSTRATE

Conc. BA mmoles/ml	Decrease of inorganic P (μmole/mg N per hr)	O_2-uptake (μ atom/mg N per hr)	Inhibition of O_2-uptake (%)	P/O ratio
		Experiment 1		
—	147·7	60·5		2·44
1×10^{-3}	neg.	25·4	58	—
5×10^{-4}	neg.	19·2	68	—
1×10^{-4}	neg.	10·8	82	—
		Experiment 2		
—	152·6	54·6		2·79
1×10^{-4}	neg.	22·8	59	—
5×10^{-5}	neg.	19·7	64	—
1×10^{-5}	neg.	18·7	66	—
		Experiment 3		
—	137·6	38·8		3·55
1×10^{-5}	neg.	7·4	81	—
5×10^{-6}	neg.	6·7	83	—
1×10^{-6}	93·2	31·1	20	3·0
		Experiment 4		
—	190·7	59·4		3·21
$5{\cdot}3 \times 10^{-6}$	neg.	13·5	77	—
$3{\cdot}3 \times 10^{-6}$	neg.	19·5	67	—
$2{\cdot}0 \times 10^{-6}$	neg.	17·0	71	—
$6{\cdot}7 \times 10^{-7}$	173·7	62·3	−5	2·79

All vessels contained phosphate buffer, pH 7·4, 90 μmoles; malonate, 30 μmoles; NaF, 60 μmoles; $MgCl_2$, 30 μmoles; α-KG, 30 μmoles; glucose, 65 μmoles; ATP, 5 μmoles; cytochrome-*c*, 0·04 μmoles; versene, 6 μmoles; hexokinase, 0·05 ml; mitochondria, 1 ml; total volume, 3 ml. Concentration of mitochondria suspension in mg N/ml, exp. 1, 0·296; exp. 2, 0·395; exp. 3, 0·197; exp. 4, 0·258. All measurements were made at 30 °C.

An explanation of the first result (a) was sought in a contamination of the BA preparation with fatty acids. If these fatty acids should be oxidized by the mitochondria an increasing oxygen consumption could be expected with increasing concentrations of inhibitor, resulting in an apparent decrease of the percentage inhibition, the inhibition of the α-ketoglutaric acid dehydrogenase having reached its maximum value at a lower BA concentration. Incubation of BA in substrate amounts with mitochondria did not give oxygen uptake, but when oxaloacetic acid was added as a sparker for fatty acid oxidation an oxygen consumption proportional to the concentration of BA and consequently to the concentration of the contaminating fatty acids was observed (Table 5). The question, however, had then to be answered how sparking was

TABLE 5. OXIDATION OF FATTY ACIDS, PRESENT AS IMPURITIES IN BONGKREKIC ACID PREPARATIONS, SPARKED BY OXALOACETIC ACID

Added BA μmoles	Oxygen consumption (μl/mg N per hr)	
	5 μmole oxaloacetic acid	10 μmole oxaloacetic acid
0	44·4	82·1
1·7	74·2	199·8
3·4	108·2	240·0
5·1	178·1	—
6·8	169·6	236·5

The reaction medium contained phosphate buffer pH 7·4, 90 μmoles; NaF, 60 μmoles; $MgCl_2$, 30 μmoles; ATP, 5 μmoles; cytochrome-*c*, 0·04 μmoles; versene, 6 μmoles; mitochondria, 1 ml. Incubation temperature, 30 °C. The total volume of the reaction medium was 3·0 ml.

effected in the experiments with α-KG as a substrate, in which malonic acid was added to inhibit succinic acid dehydrogenase. If the concentration of malonic acid used was not large enough to give a complete inhibition of succinate oxidation, sparker formation might still occur and in that case an effect of the concentration of malonic acid on the inhibition-percentage should be demonstrable. This proved actually to be the case as is shown in Table 6; it also follows from this table that the percentage inhibition of the oxygen-uptake decreases with increasing incubation time (last column excepted). This is presumably the result of a steadily increasing concentration of sparker for fatty acid oxidation. When the concentration of malonic acid was raised to 40 μmole/ml a fairly constant inhibition-percentage was obtained between 10^{-3} M and 3×10^{-6} M BA (Table 7). The concentration range between zero and maximal effect of BA seems to be very narrow, as may be concluded from Table 4 (experiment 4) and Table 7 (experiment 1).

Experiments in which succinate was used as a substrate, showed that inhibition of oxygen uptake was not a general aspect of bongkrekic acid activity: the oxidation of succinate is considerably stimulated. In a typical experiment an increase of 65 % was found with a concentration of 3×10^{-6} M bongkrekic acid. Phosphorylation however is totally abolished even with the lowest concentration bongkrekic acid used (7×10^{-7} M). The stimulating effect on the succinate oxidation is in agreement with the results of the experiment with heart muscle homogenate, where the addition of succinate could repair the strongly inhibited oxidation of endogenous substrate (Fig. 2).

TABLE 6. EFFECT OF MALONATE CONCENTRATION AND INCUBATION TIME ON THE PERCENTAGE INHIBITION OF α-KETOGLUTARIC ACID OXIDATION BY BONGKREKIC ACID

Incubation time in min	Inhibition of oxygen uptake (%)					
	Experiment 1			Experiment 2		
	No malonate	0·003 M malonate	0·02 M malonate	No malonate	0·01 M malonate	0·02 M malonate
10	54·4	59·4	72·7	61·6	69·7	72·5
20	53·2	56·1	68·5	59·7	66·9	76·8
30	53·1	53·6	67·2	57·6	64·4	76·9
40	50·8	52·0	65·8	54·6	61·1	76·9
50	45·3	49·3	63·4	47·6	49·0	71·9

The reaction medium consisted of phosphate buffer pH 7·4, 90 μmoles; NaF, 60 μmoles; versene, 6 μmoles; α-KG, 30 μmoles; $MgCl_2$, 30 μmoles; cytochrome-*c*, 0·04 μmoles; ATP, 5 μmoles; malonic acid as indicated; BA, 10^{-4} M; mitochondria, 1 ml. In experiment 1 the nitrogen content of the mitochondria suspension was 0·304 mg N/ml, in experiment 2, 0·465 mg N/ml. Incubation temperature, 30 °C.

TABLE 7. INHIBITION OF α-KETOGLUTARIC ACID OXIDATION BY VARIOUS CONCENTRATIONS OF BONGKREKIC ACID IN THE PRESENCE OF A HIGH CONCENTRATION OF MALONIC ACID

Conc. BA (mmoles/ml)	Experiment 1		Experiment 2	
	Oxygen uptake (μl/mg N per hr)	Inhibition (%)	Oxygen uptake (μl/mg N per hr)	Inhibition (%)
0	470	0	471	0
6×10^{-7}	477	−2	—	—
1×10^{-6}	463	2	—	—
$1{\cdot}5 \times 10^{-6}$	436	7	—	—
3×10^{-6}	227	52	—	—
6×10^{-6}	208	56	—	—
1×10^{-5}	196	59	224	52
3×10^{-5}	208	56	—	—
5×10^{-5}	—	—	220	53
1×10^{-4}	204	57	215	54
5×10^{-4}	—	—	216	54
1×10^{-3}	215	54	206	56

Composition of incubation medium see Table 6. Mitochondria concentration, exp. 1, 0·487 mg N/ml; exp. 2, 0·480 mg N/ml. Incubation temperature, 30 °C.

Pyruvic acid was rapidly oxidized by the heart muscle mitochondria in the presence of a small amount of succinate; this oxidation however was impaired when BA was added to the reaction medium. This is shown in Fig. 3.

The oxidation of α-ketoglutaric acid and pyruvic acid shows a great analogy: both substrates are oxidized via an acylcoenzyme A intermediate, and the hydrogen is transferred via lipoic acid to DPN. In the oxidation of succinate DPN is not involved. Therefore it seemed of interest to investigate the effect of BA on the oxidation of those intermediates of the tricarboxylic acid cycle where the hydrogen atoms are transferred via DPN, but where no acylcoenzyme A intermediate is involved. Malic acid fulfils this condition and it was found that BA inhibits the oxidation of this tricarboxylic acid cycle intermediate by heart muscle mitochondria.

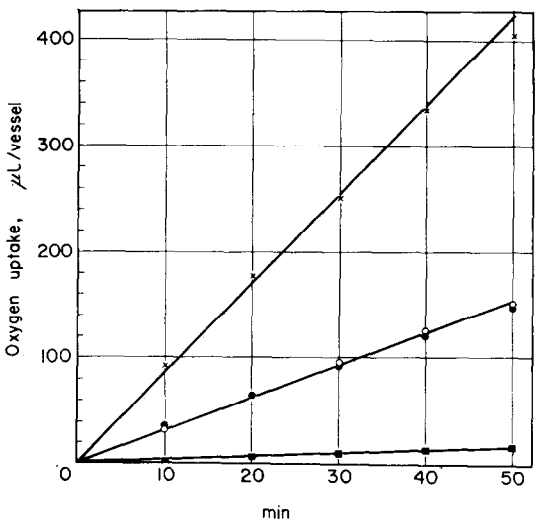

FIG. 3. Inhibition of pyruvate oxidation by BA. All Warburg vessels contained phosphate buffer pH 7·4, 90 µmoles; NaF, 60 µmoles; $MgCl_2$, 30 µmoles; cytochrome-*c*, 0·04 µmoles; versene, 6 µmoles; mitochondria 1·0 ml (0·598 mg N/ml); ATP, 5 µmoles. ■–■–■–■, succinate, 2 µmoles; ×–×–×, succinate + pyruvate (20 µmoles); ●–●–●–●, succinate + pyruvate (20 µmoles) + BA (5 × 10^{-6} M); ○–○–○–○, succinate + pyruvate (20 µmoles) + BA (1 × 10^{-5} M).

Further experiments were performed with the immediate precursor of malic acid in the cycle fumaric acid. The oxidation of fumaric acid and also the phosphorylation coupled with this oxidation proved to be very sensitive to BA. Concentrations of inhibitor as low as 6·7 × 10^{-6} M already give an inhibition of the oxygen uptake of about 40 % but there is a considerable variation in the amount of inhibition between single experiments, probably related to the mitochondria concentration.

TABLE 8. INHIBITION OF FUMARATE OXIDATION BY BONGKREKIC ACID

Conc. BA mmoles/ml	Inhibition (%)	
	Experiment 1	Experiment 2
1·3 × 10^{-6}	2	—
4 × 10^{-6}	39	—
6·7 × 10^{-6}	38	42
1·1 × 10^{-5}	42	—
1·3 × 10^{-5}	42	48·5
2 × 10^{-5}	48	51
2·7 × 10^{-5}	71	57
4 × 10^{-5}	—	66
5·4 × 10^{-5}	—	68
6·7 × 10^{-5}	—	72

Composition of incubation medium phosphate buffer pH 7·4, 90 µmoles; NaF, 60 µmoles; versene, 6 µmoles; $MgCl_2$, 30 µmoles; fumarate, 30 µmoles; ATP, 5 µmoles; cytochrome-*c*, 0·04 µmoles; mitochondria, 1 ml; total volume, 3 ml. Incubation temperature, 30 °C. Mitochondrial concentration, 0·463 mg N/ml (exp. 1) and 0·512 mg N/ml (exp. 2).

In Table 8 the results of two experiments are summarized, covering a BA-concentration range of from 1·3 × 10^{-6} to 6·7 × 10^{-5} M. Phosphorylation is totally abolished at

BA-concentrations higher than $6{\cdot}7 \times 10^{-6}$ M. At lower concentrations there is no clear relation between inhibition of O_2-uptake and phosphorylation, as may be seen from Table 9. At the lowest concentration a small rise in the P/O-ratio can be observed, but when the BA-concentration is doubled a further increase in P/O-ratio is found in one experiment and a decrease in the other.

TABLE 9. INHIBITION BY BONGKREKIC ACID OF OXIDATIVE PHOSPHORYLATION WITH FUMARATE AS SUBSTRATE

Conc. BA mmoles/ml	Experiment 1			Experiment 2		
	Percentage inhibition of		P/O ratio	Percentage inhibition of		P/O ratio
	Oxygen uptake	Phosphorylation		Oxygen uptake	Phosphorylation	
$6{\cdot}7 \times 10^{-7}$	31	22	1·96	12	6	1·92
$1{\cdot}3 \times 10^{-6}$	33	8	2·24	17	24	1·66
$6{\cdot}7 \times 10^{-6}$	60	70	1·22	52	100	—

Composition of incubation medium, see Table 8; in these experiments were also added hexokinase, 0·05 ml and glucose, 65 μmoles to each vessel. Mitochondrial concentration, 0·593 mg N/ml (exp. 1) and 0·426 mg N/ml (exp. 2). P/O ratios without BA, 1·65 (exp. 1) and 1·81 (exp. 2).

Finally some experiments were done to investigate the effect of BA on the oxidation of β-hydroxybutyrate by heart muscle mitochondria. No concomitant phosphorylation was measured and glucose plus hexokinase were therefore left out of the medium. The oxidation of this substrate was found to be very slow and it could not be enhanced by adding small amounts of succinate. This is shown in Fig. 4: the oxygen uptake of BOH + succinate (curve D) is equal to the sum of oxygen uptakes when BOH or succinate are added separately (curve A and B). BA has only a very small inhibitory effect on the oxidation of BOH alone (curve C), but a very pronounced stimulation of oxygen consumption is obtained in the presence of succinate (curve E and F).

3 *Experiments with isolated α-KG oxidase*

The enzymic oxidation of α-KG to succinic acid in mitochondria is a rather complex one. Through the work of Kaufman *et al.*[18, 19] and Sanadi *et al.*[20–24] it is known that the reaction sequence can be written as follows:

(1) α-KG + CoA-SH + DPN^+ ⟶ succinyl-S-CoA + CO_2 + DPNH + H^+

(2a) Succinyl-S-CoA + P_i + GDP ⟶ succinate + CoA-SH + GTP

(2b) GTP + ADP ⟶ GDP + ATP

(3) Succinyl-S-CoA + H_2O ⟶ succinate + CoA-SH.

All these reactions are catalyzed by separate enzymes; α-KG dehydrogenase for reaction (1), GDP phosphorylation enzyme for reaction (2a). Reaction (2b) is catalyzed by a diphosphonucleosidekinase (NDP-kinase) and reaction (3) by succinyl-CoA deacylase. GDP phosphorylation enzyme and NDP-kinase together have been called P-enzyme by Kaufman.[18] Isolated α-KG dehydrogenase contains bound thiamine-pyrophosphate and α-lipoic acid, the latter is most probably bound on the enzyme as the acid amide[25] and plays a role not only in electron transport between substrate and DPN^+ but also in acylgroup transfer to CoA.[26] Reaction (2a) seems to be the sum of

at least two reactions, since Smith *et al.*[27] could show that in *E. coli* succinate activation (reverse of reaction) proceeds via phosphoryl-S-CoA; it remains to be demonstrated that this intermediate is also involved in animal α-KG oxidase. IDP and ITP can replace GDP and GTP in reactions (2a) and (2b), as was shown by Sanadi *et al.*[24]

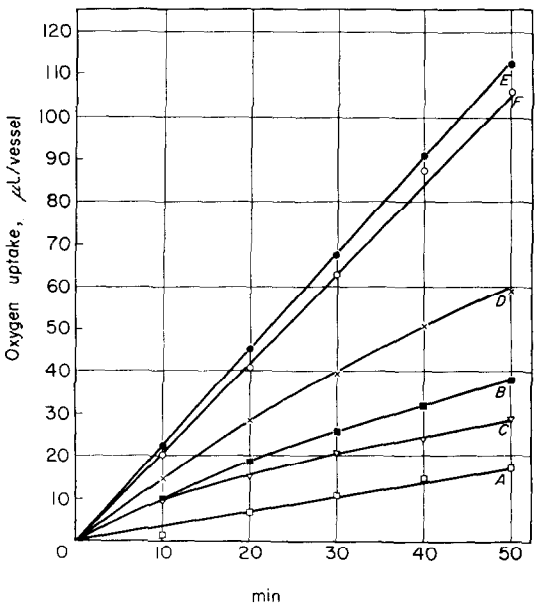

FIG. 4. Stimulation of the oxidation of β-hydroxybutyric acid by bongkrekic acid in the presence of small amounts of succinate. The vessels contained: phosphate buffer, pH 7·4, 90 μmoles; NaF, 60 μmoles; $MgCl_2$, 30 μmoles; cytochrome-*c*, 0·04 μmoles; versene, 6 μmoles; ATP, 5 μmoles; mitochondria, 1 ml and when present succinate, 2 μmoles; BOH, 40 μmoles.
Curve A: succinate, curve B: BOH, curve C: BOH + BA (10^{-5} M), curve D: BOH + succinate, curve E: BOH + succinate + BA (5×10^{-6} M), curve F: BOH + succinate + BA (10^{-5} M). Mitochondrial suspension contained 0·598 mg N/ml. Temperature, 30 °C.

When α-KG dehydrogenase was tested on its sensitivity to BA no inhibition was found, but on coupling α-KG dehydrogenase with P-enzyme an inhibition of enzymic activity was clearly demonstrated; the concentration of BA however necessary to demonstrate inhibition was very high: $1{\cdot}2 \times 10^{-3}$ M gave a decrease of 58 % of the control value (Fig. 5, curve A). When ADP was left out of the incubation medium, the oxidation velocity of α-KG was lower, but still sensitive to BA (curve B); addition of BA at the beginning of the experiment gave an initial reaction velocity, that was equal to that after BA addition at 22 min, but levelled off to zero after about 20 min (curve C). When no ADP is added reaction (2b) does not progress; as inhibition was found in this case too, it seems probable that BA attacks on the GDP (IDP) phosphorylation enzyme rather than on the NDP kinase. This can also explain the fact that with mitochondria a total inhibition of α-KG oxidation was never found: if it may be assumed that the breakdown of succinyl-CoA via reaction (3) is not impaired, oxidation of α-KG to succinate remains possible.

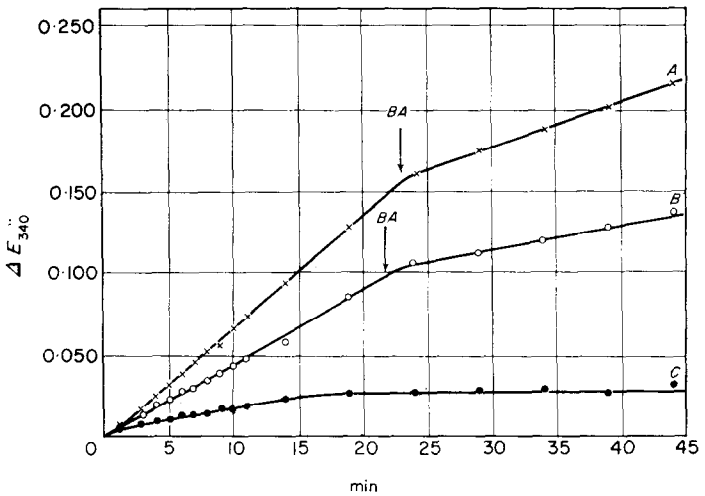

FIG. 5. Inhibitory effect of bongkrekic acid on purified P-enzyme. Beckman spectrophotometer culvettes contained 100 μmole Trisbuffer pH 7·4; 30 μmoles potassium phosphate pH 7·4; 7·5 μmoles $MgCl_2$; 0·22 μmoles DPN; 40 γ CoA; 8·3 μmoles cysteine; 25 μmoles α-KG; 1·8 μmoles IDP; 1·6 μmoles ADP; 0·2 ml α-ketoglutaric dehydrogenase; 1·0 ml P-enzyme; water to 3 ml. The cysteine was neutralized just before starting the assay. Curve A: complete reaction mixture, BA added after 23 min; curve B and C: ADP omitted from the reaction mixture, BA added after 22 min (B) or before addition of enzyme (C).

DISCUSSION

This investigation reveals that BA, must be considered as a potent inhibitor of mitochondrial enzymes. The process of oxidative phosphorylation—the energy generating mechanism in the oxidative breakdown of the carbohydrates and fats—is strongly impaired by BA, and this must lead to a lack of "energy rich" triphosphates in the BA sensitive organs.

Several phenomena that are found on BA intoxication can be explained by this general picture of the action of BA. As the phosphorylation of ADP coupled with the oxidation of 3-phosphoglyceraldehyde is the only way open to form ATP when the tricarboxylic acid cycle is blocked, it is understandable that stocks are mobilised, i.e. that glycogen disappears from liver, heart and muscle; that the glucose content of the blood is initially increased, but decreases as soon as the glycogen is exhausted; and that lactic acid will accumulate. Von Holt[6] found a very sudden appearance of shock after administration of BA to rats, which might perhaps be ascribed to heart insufficiency caused by a shortage of ATP required for performing work. In advanced cases of BA intoxication patients get in coma, which may be result of disturbance of oxidative phosphorylation in the brain.

This study sheds no light on the precise mode of action of this toxic agent; only points of attack are located. We do not know for example whether BA affects the structural integrity of the mitochondria or whether its activity may be due to inhibition of "non-structure-related" mitochondrial reactions. The first possibility seems a more plausible one, because the concentration which is effective on soluble α-KG oxidase is many times greater than with mitochondria.

The effect of BA on the oxidative capacity of mitochondria is dimorphic: the oxidation of pyruvate, α-KG and malate is inhibited, that of succinate and BOH is stimulated. In the experiments with BOH a racemic mixture of the D(−) and L(+)-form was used. As is shown by Beechey[28] both isomers are not oxidized in the same manner by rat heart mitochondria. For the oxidation of D(−)-BOH to water and carbon dioxide no catalytic amount of citric acid cycle intermediates is required as sparker, in contrast to that of L(+)-BOH, which showed a need for small quantities of fumarate, and proved to be sensitive to fluoride. Uncoupling agents like DNP and dicoumarol inhibited the oxidation of both isomers. This situation is in a sharp contrast to what is known of the oxidation of BOH by liver mitochondria. Lehninger and Greville[29] found that the sole oxidation product of D(−)-BOH was acetoacetic acid, whereas L(+)-BOH gave chiefly citric acid and only small amounts of acetoacetic acid in the presence of sparker. DNP stimulates the oxidation of BOH by liver mitochondria, as was shown by Judah.[30] In our case no promotion of oxygen uptake was found on addition of succinate; a possible explanation may be that the fluoride concentration we used was so high that sparking of L(+)-BOH oxidation was no longer possible, but then it is not understandable why the stimulation of oxygen uptake was found in the presence of succinate only. Appropriate controls ruled out the possibility that the extra oxygen consumption was due to an effect of BA on succinate and not on BOH; moreover, stimulation was also found when not succinate but oxaloacetate was added in sparking amounts. This question cannot be solved before more experimental evidence has been obtained.

The fact that BA only stimulates the oxidation of succinate and BOH may be connected with the findings of Lehninger and Cooper[31] and of Kielley and Bronk[32] who prepared fragments of mitochondria, carrying part of the oxidative and phosphorylative capacities of whole mitochondria. The only dehydrogenases still present in these particles are those for BOH and succinate and this points to a close spatial arrangement of these dehydrogenases in the intact mitochondria. This may be used as another argument for the hypothesis that BA acts primarily on the mitochondrial structure.

BA can be considered as a new type of uncoupler of oxidative phosphorylation; its structure (as far as it is known at this moment) is very uncommon and it differs principally from all other known uncouplers in that its unihibitory power includes the substrate-linked phosphorylation of α-KG.

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REFERENCES

1. W. K. MERTENS and A. G. VAN VEEN, *Geneesk. Tijdschr. Ned.-Ind.* **73**, 1223 and 1309 (1933).
2. W. K. MERTENS and A. G. VAN VEEN, *Meded. Dienst Volksgezondh. Ned.-Ind.* **22**, 209 (1933).
3. A. G. VAN VEEN and W. K. MERTENS, *Rec. Trav. Chim. Pays-Bas* **53**, 257 (1934).
4. D. H. NUGTEREN and W. BERENDS, *Rec. Trav. Chim. Pays-Bas* **76**, 13 (1957).
5. A. G. VAN VEEN and W. K. MERTENS, *Arch. Neerl. Physiol.* **21**, 73 (1936).
6. C. VON HOLT, Personal communication.
7. A. G. VAN VEEN, *Docum. Neerl. Indones. Morb. Trop.* **2**, 185 (1950).
8. H. A. KREBS and L. V. EGGLESTON, *Biochem. J.* **34**, 442 (1940).
9. K. W. CLELAND and E. C. SLATER, *Biochem. J.* **53**, 547 (1953).
10. L. BERGER, M. W. SLEIN, S. P. COLOWICK and C. F. CORI, *J. Gen. Physiol.* **29**, 379 (1946).

11. D. KEILIN and E. F. HARTREE, *Manometric techniques and tissue metabolism*, p. 211, Burgess Publishing Co., Minneapolis (1949).
12. E. MARGOLIASH, *Biochem. J.* **56**, 529 (1954).
13. S. KAUFMAN, *Methods in Enzymology* Vol. I, p. 714, Academic Press, New York (1955).
14. R. MARKHAM, *Biochem. J.* **36**, 790 (1942).
15. H. HOLZER, E. HOLZER and G. SCHULTZ, *Biochem. Z.* **326**, 385 (1954/1955).
16. E. C. SLATER and K. W. CLELAND, *Biochem. J.* **53**, 557 (1953).
17. E. C. SLATER and F. A. HOLTON, *Biochem. J.* **56**, 28 (1954).
18. S. KAUFMAN, *Phosphorus Metabolism* Vol. I, p. 370, Johns Hopkins Press, Baltimore (1951).
19. S. KAUFMAN, C. GILVARG, O. CORI and S. OCHOA, *J. Biol. Chem.* **203**, 869 (1953).
20. H. HIFT, L. OUELLET, J. W. LITTLEFIELD and D. R. SANADI, *J. Biol. Chem.* **204**, 565 (1953).
21. D. R. SANADI, D. M. GIBOSN, P. AYENGAR and L. OUELLET, *Biochim. Biophys. Acta* **13**, 146 (1954).
22. P. AYENGAR, D. M. GIBSON and D. R. SANADI, *Biochim. Biophys. Acta* **13**, 309 (1954).
23. D. R. SANADI, D. M. GIBSON and P. AYENGAR, *Biochim. Biophys. Acta* **14**, 434 (1954).
24. D. R. SANADI, D. M. GIBSON and M. JACOB, *J. Biol. Chem.* **218**, 505 (1956).
25. D. R. SANADI and R. L. SEARLS, *Biochim. Biophys. Acta* **24**, 220 (1957).
26. D. R. SANADI, M. LANGLEY and F. WHITE, *Biochim. Biophys. Acta* **29**, 218 (1958).
27. R. A. SMITH, J. F. FRANK and I. C. GUNSALUS, *Fed. Proc.* **16**, 251 (1957).
28. R. B. BEECHEY, *Biochem. J.* **66**, 10P (1957).
29. A. L. LEHNINGER and G. D. GREVILLE, *Biochim. Biophys. Acta* **12**, 188 (1953).
30. J. D. JUDAH, *Biochem. J.* **49**, 271 (1951).
31. C. COOPER and A. L. LEHNINGER, *J. Biol. Chem.* **219**, 489 (1956).
32. W. W. KIELLEY and J. RAMSEY BRONK, *J. Biol. Chem.* **230**, 521 (1958).