SUMMARY

1. Chromatography on silica gel, combined with infrared spectrometry, has been used to analyse mixtures of bile acids.

2. The results obtained by applying this method to an ox bile-salt preparation are described. Methyl lithocholate (methyl 3-hydroxycholesterol) (1.8 mg.), methyl deoxycholesterol (methyl 3:12-dihydroxycholesterol) (38 mg.), methyl chenodeoxycholesterol (methyl 3:7-dihydroxycholesterol) (0.6 mg.) and methyl cholate (methyl 3:7:12-trihydroxycholesterol)

(181 mg.) were recovered from 250 mg. of crude mixed esters.

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Enzyme inhibitions by Snake Venoms

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Snake venoms are complex mixtures of components, many of which are known to be proteins having enzymic activities. Their enormous toxic power is illustrated by the fact that the minimum lethal dose for mice of a purified preparation of neurotoxin from cobra venom is 9.0–0.03 µg./g. weight of animal (Micheel & Jung, 1936). The complex nature of venoms gives rise to a variety of symptoms whose dominant clinical features have caused the venoms to be classified as neurotoxic, haemotoxic or depressant.

Although the presence of proteolytic enzymes in venoms was described by de Lacerda (1881), it was only after an investigation of the coagulating effect of some varieties of venoms (viperine) (Lamb, 1901; Noc, 1904; Martin, 1905) had stimulated the study of venom proteases, and after Delezenne & Ledebt (1911a, b) had correlated the presence of lecithinase with haemolysis, that it became evident that enzymes may play a significant role in the pathogenic effects of venoms. Feldberg & Kellaway (1937, 1938) have pointed out that the effects of cobra venom on the circulatory system may be

accounted for, to some extent, by the presence of a phosphatidase.

A considerable variety of enzymes is now known to exist in snake venoms. These are proteolytic enzymes, phosphatidases, hyaluronidase, amino-acid oxidase, phosphoesterases, 5-nucleotidase, ribonuclease and deoxyribonuclease, cholinesterase, adenosinetriphosphatase and diphosphopyridine nucleotidase. (For review see Zeller, 1951.) How far these enzymes, or other components of venoms, are implicated in the production of neurological lesions by venoms is still quite obscure.

The variety of symptoms produced by venoms on the nervous system has been attributed to components loosely referred to as 'neurotoxins'. Most investigations have pointed to the protein nature of neurotoxins. A long time ago Mitchell (1860) separated a protein fraction from rattlesnake venom which was toxic to pigeons. Slotka & Fraenkel-Conrat (1938) reported the isolation of a neurotoxin of *Crotalus terrificus terrificus* in crystalline form which they termed 'crotoxin'. This protein has been found to possess phosphatidase activity and to

be homogeneous by electrophoresis (Li & Fraenkel-Conrat, 1942) and by the ultracentrifuge (Gralén & Svedberg, 1938).

The object of the present investigation was to discover whether the neurotoxic effects due to cobra venom might result from a disturbance of enzyme activities in the nervous system. There are, however, so many factors in venom capable of influencing its course of action that it is difficult to assess which, if any, of these factors is essential for toxicity. Investigations by Chain (1939), Ghosh & Chatterjee (1948), Fleckenstein, Tippelt & Kroner (1950) and by Fleckenstein & Gayer (1950) have shown that crude venom of a variety of snakes inhibits the activities of several dehydrogenases and oxidizing systems, but it is difficult to determine which factor is primarily involved in these inhibitions. It has, therefore, been considered necessary, in the work to be described, to treat the venom in such a way that, whilst retaining neurotoxic effects, the problem of interpretation is simplified by removal of a large variety of enzymes.

It has been known since the pioneer studies of Mitchell (1860) that when venom is heated to 100° for 30 min. it still retains toxic properties. A sample of cobra venom (from the Haffkine Institute, Bombay, India) heated for 15 min. in a boiling-water bath and tested for toxicity by intramuscular injections in mice, is lethal. For example, 50 µg. of heated venom in NaCl (0.9%) injected into six mice, killed the animals in 4 hr. Since heating the venom for 15 min. at 100° does not destroy the toxicity of cobra venom to animals, it became important to discover what enzymic activity might still remain in the heated venom.

METHODS AND MATERIALS

The venoms employed were five in number: Russell's viper venom supplied by the Haffkine Institute; Russell's viper venom obtained from Wellcome Research Laboratories, London; *Agkistrodon piscivorus* venom supplied by Ross Allen's Reptile Institute, Silver Springs, Florida; cobra venom (*Naja naja*) obtained from the Haffkine Institute; and cobra venom supplied by Dunning Inc., Baltimore. Most of the work to be described was carried out using cobra venom supplied by the Haffkine Institute. The heated venom consisted of the supernatant obtained after removing the protein coagulated on heating a solution of the crude lyophilized venom in a boiling-water bath for 15 min. The heat-treated product, prepared as described above, on drying, contained 8% N, and therefore had a protein concentration of approx. 50%.

Experiments were carried out to investigate the enzymes of heated and unheated venom and the action of heat-treated venom on the metabolism (respiration, glycolysis) of tissue homogenates. A series of isolated enzyme systems was also examined, using the usual Warburg technique.

L-Amino-acid oxidase. Measurements of O₂ uptake obtained after addition of 0.02M-L-leucine to solutions of

venom in phosphate buffer, pH 7.4, were made (Zeller, 1948).

Cholinesterase. Rates of hydrolysis of acetylcholine on addition to venom dissolved in 0.025M-NaHCO₃ were measured manometrically (Quastel, Tennenbaum & Wheatley, 1936).

Choline acetylase. Experimental details as given by Harpur & Quastel (1949) were followed.

Choline dehydrogenase. Preparations of tissue and determinations of activity were carried out according to Mann & Quastel (1937).

Pyruvic dehydrogenase of brain. Measurements of the activity of this enzyme in a brain homogenate were made with the use of the ferricyanide technique in presence of pyocyanin as carrier (Michaelis & Quastel, 1941). The homogenates were prepared by the following procedure: soon after removal from the animal, the brain was sliced, the slices were well washed in 30 ml. of 0.9% NaCl, and then minced for 1 min. in a small quantity of 0.9% NaCl.

Pyruvic oxidase of pigeon-breast muscle. This enzyme was prepared according to Stumpf, Zarudnaya & Green (1947), and its activity determined by the ferricyanide technique (Quastel & Wheatley, 1938).

Glucose oxidase of acetone-dried pigeon liver. Acetone-dried powders were prepared as follows: six fresh pigeon livers were cut up with scissors and ground in a Waring Blendor with a small quantity of water (50 ml.). The paste was added to ice-cold acetone (20 vol.). The mixture was allowed to stand for about 20 min. and filtered by suction. The powder obtained was further treated with 200 ml. of ice-cold acetone, filtered by suction and dried quickly in air by spreading over filter paper. The dried powder was kept in a vacuum desiccator in the refrigerator. Acetone-dried pigeon-liver powder 500 mg. was extracted with 10 ml. water for 2 hr. in the cold. The extract, after squeezing through muslin, was centrifuged at 3000 rev./min. The clear centrifugate was dialysed overnight at 5° against 400 ml. distilled water. The activity of the glucose dehydrogenase in the dialysate was determined in the presence of added diphosphopyridine nucleotide by the ferricyanide technique (Michaelis & Quastel, 1941). To 1 ml. of dialysed extract of the pigeon liver (equivalent to 50 mg. of the powder) 1 mg. DPN (diphosphopyridine nucleotide) and glucose and NaHCO₃ were added equivalent to a final concentration of 0.05 and 0.025M respectively. Venom was then added to the system which was incubated at 37° in 93% N₂ and 7% CO₂ (v/v) for 60 min. before addition of the ferricyanide from the side arm of the manometric vessel.

Lactic and malic dehydrogenases. These enzymes, in brain homogenates, were examined anaerobically by the ferricyanide technique.

Diphosphopyridine nucleotidase (DPN-ase). The technique of Mann & Quastel (1941) was followed.

Succinic dehydrogenase. The activity of this enzyme was determined by the ferricyanide technique.

Glucose oxidase of fungi (notatin). The technique of Keilin & Hartree (1948) was adopted.

Pyruvic dismutation system. Acetone-dried pigeon-liver powder was used as the source of the enzyme system. The pyruvic dismutase activity was determined manometrically by measuring CO₂ evolved when sodium pyruvate solution was added to a medium containing the enzyme system and NaHCO₃ in an atmosphere of 7% CO₂ and 93% N₂ at 37°. The medium contained the following constituents: NaHCO₃,

(0.025 M); phosphate buffer, pH 7.4 (0.02 M); MgCl_2 (0.01 M); DPN (1 mg./vessel); sodium pyruvate (0.02 M); heated venom (300 $\mu\text{g.}/\text{ml.}$). When venom was added the system containing the venom and enzyme was incubated for 60 min. before addition of the pyruvate.

Acetylation of sulphanilamide in presence of added adenosinetriphosphate (ATP). Acetone-dried pigeon-liver powder was used. The medium employed, when the acetylation of sulphanilamide was determined in presence of added ATP, contained the following constituents: sodium phosphate buffer, pH 7.4 (0.02 M); NaHCO_3 (0.028 M); MgCl_2 (0.01 M); NaF (0.02 M); ATP (0.003 M); sodium acetate (0.02 M); sulphanilamide (75 $\mu\text{g.}/\text{vessel}$); pigeon-liver powder (50 mg./vessel). The experiment was carried out in manometric vessels in air at 37° for 90 min. The free sulphanilamide remaining at the end of incubation period was determined by the method of Brattan & Marshall (1939).

Acetylation of sulphanilamide in presence of a respiring system. Fresh rat brain was homogenized in a medium having the following composition: MgCl_2 (0.006 M); KCl (0.090 M); potassium phosphate buffer, pH 7.4 (0.02 M). Experiments were carried out in manometric vessels at 37° for 90 min. using air as the gas phase. The following constituents were present in each vessel: brain homogenate, 140 mg.; pigeon-liver powder, 75 mg.; sodium pyruvate (0.01 M); sulphanilamide, 80 $\mu\text{g.}$; heated cobra venom, 300 $\mu\text{g.}/\text{ml.}$

Acetylation of sulphanilamide anaerobically in presence of a pyruvate-dismutating system. Experiments were carried out in manometric vessels at 37° in an atmosphere of 7% CO_2 and 93% N_2 for 90 min. The vessels contained the following constituents: Na_2HPO_4 (0.02 M); NaHCO_3 (0.025 M); MgCl_2 (0.01 M); KF (0.05 M); DPN, 1 mg.; pyruvate (0.02 M); heated cobra venom, 300 $\mu\text{g.}/\text{ml.}$; sulphanilamide, 75 $\mu\text{g.}$; pigeon-liver powder, 75 mg.

Urease. The activity of this enzyme was determined by the manometric assay of CO_2 evolved when a solution of urea placed in the side arm of a Warburg manometric vessel was tipped into a medium containing urease and buffer. The constituents of the flasks were as follows: 0.2 ml. of 10% urea; 0.2 ml. of 400 $\mu\text{g.}/\text{ml.}$ urease (Dunning) and 0.13 M-acetate buffer (pH 5.0) to 3.2 ml.

When studies of the effect of heated venom on the respiration of tissue homogenates were carried out, a solution of the venom was added to the tissue-substrate mixture immediately after thermal equilibrium had been reached. With studies of the effects of the venom on isolated enzyme systems, the venom was incubated with the enzyme under investigation for a standard time (usually 60 min.), after which the substrate was added.

RESULTS

Activities of enzymes in crude and heated venoms

The activities of cholinesterase, ATP-ase, DPN-ase, L-amino-acid oxidase and lecithinase were estimated in crude and heated snake venoms. The results showed that, although the crude venom contains all the enzymes mentioned, only lecithinase was active in the heated venom, though to a diminished extent. Heating venom at 100° for 15 min. fully inactivates the proteolytic enzymes (Ghosh & Chaudhury, 1938), hyaluronidase (Madi-

naveitia, 1941), 5-nucleotidase (Zeller, 1950) and phosphatases (Gulland & Jackson, 1938) that are normally present. Taborda, Taborda, Williams & Elvehjem (1952) have recently shown that ribonuclease activity of *Bothrops jararaca* venom is completely lost by heating the venom to 80, 90 and 100°.

It appears probable, therefore, that of all the enzymes known to occur in crude venom only the lecithinase present retains activity after heating at 100° for 15 min.

The effect of heated cobra venom on the oxidation of various substrates by brain tissue

The tissue used was rat-brain homogenate, unless otherwise stated. The brain was removed from the animal soon after decapitation, chilled in cracked ice and homogenized in a Potter-Elvehjem homogenizer for 1 min. in 0.9% NaCl solution. A 1 ml. portion of the homogenate containing 150 mg. brain was used per vessel. The brain homogenate was suspended in phosphate-Locke solution, pH 7.4, containing the following constituents (final concentrations): Na_2HPO_4 (0.02 M); NaCl (0.13 M); KCl (0.004 M); CaCl_2 (0.002 M). The substrates were neutralized where necessary. Heated cobra venom, contained in 0.3 ml., was tipped from the side arm of the Warburg manometric vessel after thermal equilibration. O_2 uptakes were measured at 37°. The gaseous phase in the manometric vessels was air. CO_2 was absorbed by KOH in the centre wells.

The results are shown in Table 1. It is evident that heated venom inhibits the rate of oxygen uptake of the brain homogenate in the presence of a variety of substrates. The inhibition increases progressively with time. The substances investigated were glucose, fructose, pyruvate, succinate, L-glutamate and α -ketoglutarate.

The extent of inhibition and the course of the development of the effect differ with different substrates. The results shown in Table 1 give the percentage inhibitions produced by heated venom on the oxygen uptake found with various substrates after 50 and 100 min. contact of venom with brain tissue. When glucose and sodium pyruvate are used as substrates, the heated venom produces an initial small acceleration of respiration of the brain homogenate which falls off with time. This acceleration, which is always found with these substrates, is absent with the other substrates used.

Further results are recorded in Fig. 1 which show that the inhibition of the rate of oxygen uptake by rat-brain homogenate in presence of glucose decreases with diminishing concentrations of heated venom, and the initial activating effects become more pronounced. The inhibition of respiration in presence of fructose starts immediately, but the percentage inhibition produced after 100 min. is no greater than that found for glucose or pyruvate.

In the presence of sodium succinate or sodium L-glutamate the inhibitory effect of heated venom is

apparent almost immediately and increases rapidly. After 100 min. the inhibition of total oxygen uptake was 57 % for succinate and 68 % for glutamate. The oxidation of α -ketoglutarate is only feebly affected by the heated venom, the inhibition after 100 min. being only 16 %. In the absence of added substrate the heated venom produced an immediate fall in respiration which increased with time.

The exact nature of the endogenous respiration of brain is obscure. The pronounced differences obtained between the inhibitory effects of heated venom on glucose and pyruvate oxidations and the

inhibition of the residual respiration of the homogenate indicate that glucose and pyruvate are probably not endogenous substrates.

The initial accelerating effect of venom on the oxidation of glucose and pyruvate is diminished in the presence of added ATP (0.01M), which itself secures an increased rate of respiration. Possibly, the initial acceleration due to venom is caused by the liberation of adenylic acid (or a derivative of this) from the brain tissue by the action of venom. Kellaway & Trethowie (1940) found that the presence of cobra venom brings about the liberation

Table 1. *Percentage inhibition produced by heated cobra venom (300 μ g./ml.) on the oxidation of various substrates by rat-brain homogenate*

Exp.	Substrates added to brain homogenate (150 mg.)	After 50 min.		After 100 min.	
		O ₂ uptake (μ l.)	Percentage inhibition of total O ₂ uptake	O ₂ uptake (μ l.)	Percentage inhibition of total O ₂ uptake
1	Glucose (0.01M)	202	—	324	—
	Glucose + heated venom	200	Nil	260	20
	Pyruvate (0.02M)	218	—	350	—
	Pyruvate + heated venom	198	9	234	33
	Succinate (0.05M)	350	—	570	—
	Succinate + heated venom	183	48	248	57
	Nil	138	—	206	—
2	Nil + heated venom	76	45	90	56.5
	Fructose (0.02M)	150	—	285	—
	Fructose + heated venom	115	23	193	32
	α -Ketoglutarate (0.02M)	109	—	199	—
	α -Ketoglutarate + heated venom	102	6	166	16
	L-Glutamate (0.02M)	124	—	250	—
	L-Glutamate + heated venom	55	56	90	68
	Nil	90	—	150	—
	Nil + heated venom	70	33	90	40

Fig. 1. The effect of different concentrations of heated venom on glucose (0.01M) oxidation by rat-brain homogenate (100 mg.). Determinations were made as described in the text. A, brain + glucose; B, brain + glucose + heated cobra venom (100 μ g./ml.); C, brain + glucose + heated cobra venom (30 μ g./ml.); D, brain + glucose + heated cobra venom (20 μ g./ml.).

of adenylic acid and its derivatives from rabbit heart and liver.

The effects of different heated venoms on pyruvate oxidation by guinea pig brain homogenate

Different venoms were tested for their effects on the oxidation of pyruvate by guinea pig brain homogenate in case there was any pronounced difference in behaviour. The four samples tested were cobra venom (Haffkine), cobra venom (Dunning), *Agkistrodon piscivorus* venom, and Russell's viper venom (Haffkine). The first three of these belong to the neurotoxic group of venoms. Russell's viper venom has the least neurotoxic action of the venoms investigated.

The three venoms belonging to the neurotoxic group show only quantitative differences in their behaviour (Fig. 2). They produce the usual initial activation followed by progressively developing inhibition. Cobra venom (Haffkine) is the most potent, cobra (Dunning) is next in potency and the *A. piscivorus* venom has the least effect. In contrast

to the behaviour of the neurotoxic group of venoms, Russell's viper venom brings about an increased rate of respiration throughout the experimental period.

The effect of heated venom on different components of the succinic oxidase system

Since heated venom causes a pronounced inhibition of the succinic oxidase system in brain homogenates, it was of importance to study the action of heated venoms on separate components of the system. Although there may be some dispute regarding the various factors which are believed to be the intermediate links between the dehydrogenases and the cytochrome system, it is established

Fig. 2. The effects of different heated venoms (300 $\mu\text{g./ml.}$) on pyruvate (0.02M) oxidation by guinea pig brain homogenate (100 mg.). A, brain + sodium pyruvate; B, brain + sodium pyruvate + *Agkistrodon piscivorus* venom; C, brain + sodium pyruvate + cobra venom (Dunning); D, brain + sodium pyruvate + cobra venom (Haffkine); E, brain + sodium pyruvate + Russell's viper venom.

that the oxidation of succinate is accomplished through the combined action of succinic dehydrogenase, cytochrome *c*, cytochrome oxidase and oxygen. The toxic actions of heated venom on the various components of this system were studied separately. As a result of these studies it was found that venom attacked the succinic oxidase system at more than one point. Both cytochrome oxidase as well as succinic dehydrogenase were found to be inactivated by the heated venom, whilst cytochrome *c* was apparently completely resistant to its effects.

Cytochrome oxidase. Cytochrome oxidase activity was determined using neutralized *p*-phenylenediamine as substrate and rat-brain homogenate as the source of enzyme. It is seen (Fig. 3) that in 1 hr. the venom inhibited 50% of the activity of the enzyme. In 2 hr. the inactivation of the enzyme was found to

be complete. These results are in agreement with the findings of Chatterjee (1949) who has isolated from cobra venom, by salt fractionation, a substance which is inhibitory to cytochrome oxidase.

Cytochrome *c*. The effect of heated cobra venom on cytochrome *c* was investigated by observing the changes in the spectrum of reduced cytochrome *c* as measured by the

Fig. 3. Variation, with time, of the inhibitory action of heated cobra venom on cytochrome oxidase activity of rat-brain homogenate. The rat-brain homogenate (175 mg./vessel) was suspended in phosphate-Locke (see text). Substrate, *p*-phenylenediamine (20 mg./vessel). Heated cobra venom, 300 $\mu\text{g./ml.}$ Temperature, 37°. Gas phase, air. The substrate was added from the side arm of the Warburg vessel at definite intervals of time after the venom had been in contact with the enzyme. The $\mu\text{l. O}_2$ absorbed were recorded for 30 min. after adding the substrate. A, B and C, substrate added after 60, 90 and 120 min. respectively. A', B' and C', venom present, substrate added after 60, 90 and 120 min. respectively.

Beckman spectrophotometer. A solution of cytochrome *c* in 0.2M-phosphate, pH 7.4, containing 0.3 mg./ml. was incubated with heated venom prepared in the usual way for 30 min. at 37°. The final concentration of the venom was 300 $\mu\text{g./ml.}$; 3 ml. of the mixture were then added to the cell and the spectrum determined after reduction with sodium dithionite. The results showed complete identity of the spectra, before and after incubation with heated venom.

The effect of heated venom on succinic dehydrogenase

The activity of succinic dehydrogenase, measured anaerobically by the ferricyanide technique, is greatly reduced on exposure of the enzyme to heated

venom. Typical observations are shown in Fig. 4. These illustrate the relative susceptibilities of the brain and heart-muscle preparations of succinic dehydrogenase to the action of heated venom. The heart-muscle preparation is apparently more vulnerable than the brain preparation.

Determinations were made of the effects of changes in concentration of heated cobra venom on the percentage inhibitions of succinic dehydrogenase

Fig. 4. The effect of heated cobra venom on succinic dehydrogenase activity of rabbit brain and heart tissue homogenates. Medium: bicarbonate-Locke (NaHCO_3 , 0.025M; NaCl , 0.3M; CaCl_2 , 0.002M; KCl , 0.004M). A mixture (0.2 ml.) of $\text{K}_3\text{Fe}(\text{CN})_6$ and NaHCO_3 was added from the side arm. Heart-muscle or brain homogenate, 200 mg./vessel. Substrate: 0.05M-sodium succinate. A, heart muscle + sodium succinate; B, heart muscle + sodium succinate + heated venom (300 $\mu\text{g.}/\text{ml.}$); C, brain + sodium succinate; D, brain + sodium succinate + heated venom (300 $\mu\text{g.}/\text{ml.}$); E, corresponding controls without added substrate.

of rat-heart muscle. The heart-muscle preparation, in these experiments, was exposed to the venom for 1 hr. before the substrate was added. Typical results are given in Fig. 5. It is seen that the percentage inactivation due to the heated venom increases with concentration until a limiting value is reached with 150 $\mu\text{g.}/\text{ml.}$ of venom. Further increase in concentration of venom has apparently little effect on the percentage inhibition. The maximum inhibition produced under these experimental conditions was 55%. The aerobic oxidation of succinate is affected

to a greater extent by heated venom than the anaerobic system. This is probably due to the additional inactivation of cytochrome oxidase.

The protective action of succinate on the inhibition of succinic dehydrogenase by heated cobra venom

It was of interest to determine whether succinate would protect succinic dehydrogenase against the inactivating effect of heated venom. The effect of time of contact between the venom and enzyme was therefore studied in two series of experiments, one in which the succinate was present with the tissue and venom throughout the experimental period, and the

Fig. 5. The effect of different concentrations of heated cobra venom on the percentage inhibition of succinic dehydrogenase activity. Constituents of the medium were the same as those described in Fig. 4. The venom was incubated with 50 mg. rat heart-muscle homogenate and succinate (0.05M) for 60 min. before the addition of the ferricyanide from the side arm. Percentage inhibitions were determined after an experimental period of 30 min.

other in which the succinate was tipped into a mixture of venom and tissue at various intervals of time.

The results obtained are summarized in Table 2. Comparing the two series of results, it is evident that in absence of succinate the inhibitory effect of venom is greater than in its presence. Thus, succinate protects succinic dehydrogenase against the toxicity of heated venom. Apparently the heated venom attacks the enzyme protein itself and not any of the intermediate co-factors involved in the transfer of hydrogen from the dehydrogenase to the ferricyanide. Binet, Weller & Jaulnes (1937) reported that glutathione added to solutions of crude cobra venom at pH 7.4 or 8.4 considerably reduced its toxicity to animals. The toxicity of purified preparation of neurotoxin of *Naja tripudians* (a variety of cobra) was also reduced by glutathione (Micheel & Schmitz, 1938).

Both in the presence and absence of glutathione (0.01 M), venom inhibits the succinic oxidase system of a rat-brain homogenate by 65 %, after being in contact with the tissue for a total period of 2 hr. The inhibitory factor present in heated venom, therefore, is not inactivated by glutathione under the given experimental conditions.

The effect of heated venom on choline oxidase

Mann & Quastel (1937) showed that in rat liver choline oxidase catalyses the oxidation of choline to betaine aldehyde. As a result of studies on the intermediate carriers involved in this reaction Mann, Woodward & Quastel (1938) concluded that the choline dehydrogenase system consists essentially of the dehydrogenase, cytochrome c, cytochrome oxidase and oxygen. It was also shown that this dehydrogenase can reduce ferricyanide under

choline oxidase is much less than that observed in the presence of the heated venom. This observation suggests that crude venom lacks substances, other than those present in the heated venom, capable of destroying choline oxidase, and that some constituents of the crude venom interfere with choline oxidase inactivation by heated venom.

The inactivating action of heated cobra venom on choline oxidase increases with time, the amount of inactivation being similar to that found with succinic dehydrogenase.

The effect of heated venom on anaerobic glycolysis by guinea pig brain homogenate

It has been demonstrated that venom inhibits the aerobic oxidation of glucose. Since anaerobic glycolysis is an important pathway by which glucose may be broken down, it was important to

Table 2. *The protective action of succinate on the inhibition of succinic dehydrogenase by heated cobra venom*

Time interval before tipping $K_3Fe(CN)_6$	Relevant constituents of the system	With succinate present in contact with enzyme and venom		Succinate tipped with $K_3Fe(CN)_6$	
		CO ₂ evolved in 30 min. (μ l.)	Percentage inhibition (at 37°)	CO ₂ evolved in 30 min. (μ l.)	Percentage inhibition (at 37°)
20 min.	Succinate (0.05 M) + rat-heart muscle (50 mg.)	710	—	750	—
	Succinate (0.05 M) + rat-heart muscle (50 mg.) + 150 μ g./ml. heated venom	580	18.0	474	37.0
45 min.	Succinate (0.05 M) + rat-heart muscle (50 mg.)	710	—	770	—
	Succinate (0.05 M) + rat-heart muscle (50 mg.) + 150 μ g./ml. heated venom	390	45.0	356	54.5

anaerobic conditions. Furthermore, like succinic dehydrogenase, choline oxidase activity is intimately associated with the structure of the cell, and the enzyme has not yet been obtained in a soluble state. In view of the resemblance between the choline- and succinate-oxidizing systems, the action of heated venom on choline oxidase of rat liver was investigated.

Estimations of choline oxidase activity were carried out anaerobically by the manometric method using ferricyanide as the terminal hydrogen acceptor. The homogenate was incubated with the heated venom before the ferricyanide was tipped from the side arm. Typical results are given in Fig. 6.

It will be observed that heated venom causes a pronounced inhibition of choline dehydrogenase. In an experimental period of 50 min. heated venom inactivates 60 % of the enzyme. When crude venom is used (see Fig. 6), the inhibition produced on

Fig. 6. The effect of heated and crude cobra venom on choline oxidase activity of rat-liver homogenate. Medium: bicarbonate-Locke medium. Mixture of $K_3Fe(CN)_6$ and $NaHCO_3$ (0.2 ml.) added from the side arm. Temperature, 37°. Gas phase, 93 % N_2 , 7 % CO_2 . A, rat liver (100 mg.) + choline chloride (0.01 M); B, rat liver (100 mg.) + choline chloride (0.01 M) + heated venom (300 μ g./ml.); C, rat liver (100 mg.); D, rat liver (100 mg.) + choline chloride (0.01 M) + crude venom (300 μ g./ml.).

discover whether heated venom can attack the enzyme systems concerned.

Mellanby (1934) observed that, whilst the venoms of *Lachesis atrox*, *L. lanceolatus* and of viper, produced no effect on the anaerobic glycolysis by cancer tissue, cobra venom even in small doses (1 in 1500) inhibited glycolysis. In extension of these studies, Chain (1937) showed that crude venoms of Australian snakes inhibited glycolysis by rabbit-

Fig. 7. The effect of heated and crude cobra venom on glycolysis by guinea pig brain homogenate. Medium: K_2HPO_4 buffer (pH 7.4) 0.0025M; $KHCO_3$, 0.025M; $MgCl_2$, 0.006M; Na_2ATP , 0.006M; potassium hexosediphosphate, 0.0025M; sodium pyruvate, 0.005M; DPN, 0.02%; glucose, 0.01M. Enzyme: guinea pig brain homogenate (70 mg./vessel) prepared in 0.08M-nicotinamide and 0.15M-KCl. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2 . A, brain + glucose + heated venom (300 μ g./ml.); B, brain + glucose; C, brain + heated venom (300 μ g./ml.); D, brain; E, brain + glucose + crude cobra venom (300 μ g./ml.).

muscle extract and to a lesser extent fermentation. It was reported that the inhibitor in these venoms is thermostable. In the present investigation, the action of heated cobra venom on glycolysis of guinea pig brain homogenate and on brain hexokinase has been examined. The results (Figs. 7 and 8) indicate that the heated venom causes a pronounced activation of both.

Under similar experimental conditions the same quantity of crude unheated cobra venom causes complete inhibition of glycolysis doubtless owing to the presence of DPN-ase and ATP-ase.

The effect of heated venom on the hexokinase activities of brain and yeast

In order to discover whether the activation of glycolysis by heated venom was due to an accelerating effect on hexokinase activity, the action of heated venom on the hexokinase of guinea pig brain homogenate and, also, of an extract of acetone-dried yeast, was investigated. Preliminary experiments had already shown that heat-treated venom

Fig. 8. The effect of heated cobra venom on hexokinase activity of guinea pig brain homogenate. Medium: $KHCO_3$, 0.025M; KF, 0.1M; $MgCl_2$, 0.008M; iodoacetate, 0.005M; glucose, 0.01M; brain homogenate 100 mg./vessel. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2 . A, brain + ATP (0.004M) + heated venom (300 μ g./ml.); B, brain + ATP (0.004M); C, brain + ATP (0.002M) + heated venom (300 μ g./ml.); D, brain + ATP (0.002M); E, brain + heated venom; F, brain.

contains no ATP-ase. The results obtained with brain homogenate in the presence of two concentrations of ATP are shown in Fig. 8. It is seen that heated venom causes about 30% increase in hexokinase activity. Results obtained with yeast hexokinase demonstrate that the heated venom has no action on this system.

Although the experimental data are insufficient to determine the nature of the activating factor, it seems likely that the activation of brain glycolysis

by heated venom is connected with its effect on brain hexokinase activity. Furthermore, from the results obtained on the action of venom on glycolysis by brain tissue, it may be concluded that none of the series of enzymes concerned in the anaerobic breakdown of glucose to lactic acid is inactivated by heated venom.

Glucose oxidase ('notatin'). This enzyme has flavin-adenine dinucleotide as its prosthetic group (Keilin & Hartree, 1948) and is freely extractable. The enzyme is quite unaffected by the presence of heated cobra venom (Table 4, Exp. 2).

Glucose oxidase of pigeon liver. This enzyme, which also catalyses the oxidation of glucose to gluconic acid, differs from notatin in its requirement of DPN for its activity (Andersson, 1934). A preparation made from acetone-dried pigeon liver was used as the source of enzyme. Heated cobra venom has no inhibitory effect on its activity (Table 4, Exp. 1).

The results obtained with the soluble glucose-oxidizing enzymes from *Penicillium* and from pigeon liver are in contrast to those found with the glucose-oxidizing system of brain which is inhibited by the heated venom (see Fig. 1).

The effects of heated venom on the anaerobic breakdown of pyruvate

As was shown, heated venom produces an inactivation of the enzyme system involved in the aerobic oxidation of pyruvate by brain tissue. As many of the symptoms caused by venom intoxication are believed to result from lesions produced in various parts of the central nervous system, the effect of heated venom on the enzymes concerned with the metabolism of pyruvate, which is known to be involved in the functional activity of the nervous system, was studied in greater detail. Investigations reported here deal with the effect of heated venom on the anaerobic oxidation and dismutation of pyruvate by animal tissues. The results obtained demonstrate that the anaerobic oxidation of pyruvate is very susceptible to the toxic action of heated venom, whilst dismutation is completely unaffected. The anaerobic oxidation of pyruvate by brain tissue was determined by the ferricyanide technique in presence of pyocyanin as a carrier, as described by Michaelis & Quastel (1941).

The results obtained (Fig. 9) demonstrate that heated venom completely inactivates pyruvic oxidase, reducing the evolution of carbon dioxide to the level of the control without substrate. Crude venom was found to produce a similar inhibition. The inhibition produced by heated venom on the anaerobic system increases progressively with time. In this respect it resembles the inhibition produced by heated venom on the aerobic oxidation of pyruvate.

The results of experiments to determine the effect of changes of concentration of venom are shown in Fig. 10. It is evident that the percentage inhibition increases with concentration of the inhibitor until it reaches a maximum of 100% with 75 μ g. of venom protein per ml. About 12 μ g. of venom protein per ml. are sufficient to inactivate 50% of this enzyme system.

Fig. 9. The effect of heated cobra venom (300 μ g./ml.) on the anaerobic oxidation of pyruvate (0.02 M) by rat-brain homogenate. Medium: 0.025 M bicarbonate-Locke medium + 0.3 ml. pyocyanin (3.2 mg./100 ml.). Mixture of $K_3Fe(CN)_6$ and $NaHCO_3$ (0.2 ml.) added from the side arm. Rat-brain homogenate, 125 mg./vessel. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2 .

Fig. 10. Effects of variation of concentration of heated venom on the percentage inhibition of the anaerobic oxidation of pyruvate (0.02 M). Heated venom was incubated with the enzyme for 60 min. before the addition of the ferricyanide mixture. The percentage inhibition was calculated after an experimental period of 60 min.

Linkage of toxicity of heated venoms with enzymes associated with cell particulate matter

It is evident from the results already described with succinic, choline and pyruvic dehydrogenases that heated cobra venom inhibits, at rates that increase with time, enzymes whose activities are intimately associated with cell particulate matter. The action of heated venom on a variety of enzyme

systems active in cell-free extracts was therefore studied to discover whether the action of the venom is linked with the physical structure of the enzyme system. In this connexion, a study of the effect of heated venom on 'soluble' pyruvic oxidase was of importance.

Effects of heated venom on enzymes active in cell-free extracts

Pigeon breast. Pigeon-breast muscle has been shown to contain a 'soluble' pyruvic oxidase which catalyses the transfer of hydrogen from pyruvate to molecular oxygen or to ferricyanide under anaerobic conditions (Stumpf *et al.* 1947). This enzyme has now

Fig. 11. The effect of heated venom (300 µg./ml.) on the activity of pyruvic oxidase in an extract of pigeon-breast muscle. Medium: 0.025M-bicarbonate-Locke medium, $K_3Fe(CN)_6$ + $NaHCO_3$ mixture added from the side arm. Pigeon-breast muscle extract, 0.5 ml./vessel. Temperature, 37°. Gas phase, 93% N_2 + 7% CO_2 . A, tissue extract + sodium pyruvate (0.02M); B, tissue extract + sodium pyruvate + heated venom; C, tissue extract; D, tissue extract + heated venom.

been purified and obtained in a cell-free preparation (Jagannathan & Schweet, 1952).

The results obtained (Fig. 11) show that heated venom has no effect on the pyruvic oxidase activity of pigeon-breast muscle extract, in marked contrast to its action on the pyruvic oxidase prepared from brain tissue. The completely contrasting susceptibilities shown by these two types of pyruvic oxidase are in agreement with the view that the venom inactivates those enzymes whose activity is dependent on their components having particular spatial arrangement in the cell. These arrangements are presumably maintained by linkages which are susceptible to disruption by the venom.

Pigeon-liver acetone-dried powder. It has been demonstrated that many animal tissues, both in the form of slices (Krebs & Johnson, 1937) and minces (Weil-Malherbe, 1937), can degrade pyruvate under

anaerobic conditions by a dismutation reaction in which pyruvate acts both as a donor and acceptor of hydrogen. This reaction is catalysed by enzyme systems which are active in extracts. A study of the effects of heated venom on this system was carried out.

Acetone-dried pigeon liver was used as the source of enzyme. Its activity in dismuting pyruvate was determined manometrically by measuring the CO_2 evolved when sodium pyruvate was tipped into a medium containing the enzyme and sodium bicarbonate (atmosphere 7% CO_2 , 93% N_2 at 37°).

The effects obtained with pigeon-liver powder are shown in Table 4, Exp. 6. Heated venom has no inhibitory action on the system of enzymes involved in the dismutation of pyruvate.

Table 3. *The effect of heated venom on the acetylation of sulphanilamide*

Chief constituents of the system (+ sulphanilamide)	Sulphanilamide acetylated (µg.)	Percentage acetylation of sulphanilamide
Pyruvic oxidase system (rat-brain homogenate) + pigeon-liver powder	40.4	50
Pyruvic oxidase system + pigeon-liver powder + 300 µg./ml. heated cobra venom	17.5	22.5
Pyruvic-dismuting system (pigeon-liver powder) + 0.05M-KF	45.0	60.0
Pyruvic-dismuting system (pigeon-liver powder) + 300 µg./ml. heated cobra venom + 0.05M-KF	42.0	56.0
Pigeon-liver powder + acetate + ATP (0.003M) + NaF (0.02M)	41.0	54.5
Pigeon-liver powder + acetate + 300 µg./ml. heated cobra venom + ATP (0.003M) + Na (0.02M)	36.7	46.5

The effects of heated venom have also been studied on a larger variety of enzyme systems, both from animal and other sources, all of which have the common feature of being active in cell-free extracts. It was found (Table 4) that heated cobra venom, in concentrations of 150 µg. venom protein per ml., was without inhibitory action on the following enzyme systems: (1) lactic dehydrogenase of brain and of yeast; (2) malic dehydrogenase of brain; (3) urease—heated venom was found to produce a small inhibitory effect (of the order of 10%) on this enzyme; (4) acetylations of choline and sulphanilamide—recent observations (Braganca & Quastel, 1952) have shown that heated cobra venom exerts no inhibitory effect on the acetylation of choline by extracts of brain in presence of added ATP. The effect of heated cobra venom on the acetylation of

sulphanilamide by extracts of pigeon liver both in presence of added ATP and in presence of metabolizing pyruvate is shown in Table 3. These observations demonstrate that, when energy is supplied in the form of added ATP, the heated venom has only a negligible effect on the acetylation of sulphanilamide. Different results are obtained if the acetylation is made to depend on the respiratory activity of the brain or liver, no ATP being added to the system. If, under these conditions, the energy for the acetylation process is derived from the dismutation of pyruvate, the acetylation of sulphanilamide is not affected by the heated venom; but, when the energy is generated by the oxidation of

reported to be remarkably resistant to heat (Hughes, 1935). Since the interaction between lecithinase A of cobra venom and lecithin leads to the liberation of free acid groups, it is possible to obtain a measure of lecithinase activity using the Warburg manometric technique (Zamecnik, Brewster & Lipmann, 1947).

The lecithinase activities of crude and heated cobra venom (Haffkine) were determined using a purified sample of lecithin. The results obtained using 1 mg. of venom/vessel and varying concentrations of the lecithin are given in Figs. 12*a* and *b*. These demonstrate that with heated venom, the limiting velocity of breakdown of lecithin is reached with 40 mg. lecithin/vessel.

Table 4. *Effects of heated cobra venom on various enzyme systems active in cell-free extracts*
(Details of these experiments in text)

Exp.	Enzyme system	Relevant constituents of the system	CO ₂ evolved (μl.)	O ₂ uptake (μl.)
1	Glucose oxidase (pigeon liver)	No added substrate	40	—
		Glucose	114	—
		Glucose + heated venom	108	—
2	Glucose oxidase (notatin)	Glucose	—	470
		Glucose + heated venom	—	475
3	Lactic dehydrogenase (rat brain)	No added substrate	88	—
		No added substrate + heated venom	84	—
		Lactate	178	—
		Lactate + heated venom	181	—
4	Lactic dehydrogenase (yeast)	Sodium lactate	920	—
		Sodium lactate + heated venom	895	—
5	Malic dehydrogenase (rat brain)	Malate	108	—
		Malate + heated venom	103	—
		No added substrate	50	—
		No added substrate + heated venom	50	—
6	Pyruvic dismutase (pigeon liver)	Pyruvate	187	—
		Pyruvate + heated venom	178	—
7	Pyruvic oxidase (pigeon-breast muscle)	No added substrate	50	—
		No added substrate + heated venom	45	—
		Pyruvate	180	—
		Pyruvate + heated venom	170	—
8	Urease (400 μg.)	Urea	380	—
		Urea + heated venom	340	—

pyruvate, a pronounced inhibition of the acetylation of sulphanilamide is produced. It is evident from these findings that heated venom affects the acetylation process only indirectly through its effect on the metabolism of pyruvate.

The nature of the toxic factor in heated cobra venom

The progressive increase in inhibitory activity of heated venom with time suggests that the inhibitor may be an enzyme degrading certain essential components of cellular structure. This idea is supported by the fact that further heating in alkaline solution (pH 10.0) destroys its activity. Most enzymes present in venom are not active in the crude heated product but lecithinase A has been

Fig. 12. The lecithinase activities of (a) crude and (b) heated cobra venom. Medium: 0.025 M-bicarbonate-Locke medium. Lecithinase activity was based on μl. CO₂ evolved in 25 min. in presence of varying quantities of lecithin. Lecithin added as a solution in ethanol. Temperature, 37°. Gas phase, 93% N₂ + 7% CO₂. Venom, 1 mg./vessel.

The action of venom phospholipases on heated brain and heart tissues

Results shown in Fig. 13 indicate the effects obtained when crude and heated venoms react with brain-tissue homogenate, previously heated for 30 min. at 100°. It is evident from these observations that crude and heated venoms liberate acid groups from constituents of the brain, indicating a probable breakdown of the brain phospholipins. The course of the reaction seems to be similar to that observed with lecithin. Though crude venom is more active than the heated product, it is evident that the latter is capable of bringing about the disruption of structures in which phospholipins are concerned in the brain. It is reasonable to consider that this may be the mechanism by which the heated venom

Fig. 13. The effects of phospholipases of crude and heated cobra venoms on heated brain tissue. Medium: 0.025 M-bicarbonate-Locke medium. Experimental period, 30 min. Temperature, 37°. Gas phase, 93% N₂ + 7% CO₂. Venom, 1 mg./vessel.

inactivates a number of the enzyme systems essential for the respiratory processes. Such inactivation may also be sufficient to explain many of the toxic effects resulting from venom administration.

Russell's viper venom, which lacks the neurotoxic effects of cobra venoms, shows a much smaller lecithinase activity. Several experiments have demonstrated that its activity (after heating) is less than one-third of that of heated cobra venom.

Heated Russell's viper venom produces a smaller inhibition of succinate oxidation than that given by the heated cobra venom. The results obtained on the effects of heated Russell's viper venom and cobra venom on the overall oxidation of succinate are given in Fig. 14. This finding lends further support to the view that the inhibitory action of the heated

venom on the complete oxidation of succinate is associated with the lecithinase activity of the venom.

The effects of calcium ions on the inhibitory action of heated venom on respiratory enzyme systems

Delezenne & Ledebt (1911*a, b*) investigated the properties of lecithinase in venoms responsible for haemolysis and showed that Ca⁺⁺ had marked effects on this phenomenon. The activating effect of calcium on haemolysis by cobra venom was later confirmed by Holden (1934).

Fig. 14. The effect of heated cobra venom and heated Russell's viper venom on the oxidation of succinate by rat-brain homogenate. Medium: 0.02 M-phosphate-Locke. Temperature, 37°. Gas phase, air. Homogenate, 150 mg./vessel. A, brain + sodium succinate (0.05 M); B, brain + sodium succinate (0.05 M) + heated Russell's viper venom (300 μg./ml.); C, brain + sodium succinate (0.05 M) + heated cobra venom (300 μg./ml.).

The activating action of calcium ions on the toxic effect of heated cobra venom was shown with the system of enzymes catalysing the aerobic oxidation of glucose. The results obtained are shown in Fig. 15. It is evident that a sharp increase in the inhibitory action of the heated venom is brought about by the greater concentration of Ca⁺⁺. Furthermore, the initial lag period preceding the onset of the inhibitory effect is no longer detectable.

DISCUSSION

It is evident from the observations of the action of heated venom on a variety of enzyme systems that the inhibitor present is not a general enzyme poison. Heated venom has been found to attack the following enzyme systems to varying extents: (1) Systems contained in brain homogenate concerned with the oxidation of glucose, pyruvate, glutamate, succinate, α -ketoglutarate, and fructose. (2) Pyruvic dehydrogenase of rat brain. (3) Succinic dehydrogenase of brain and heart-muscle tissues. (4) Cytochrome oxidase of brain tissue. (5) Choline oxidase of rat liver.

Fig. 15. The effect of calcium on the inhibitory action of heated venom on glucose oxidation by rat-brain homogenate. Medium: Na_2HPO_4 buffer (pH 7.4) 0.02M; NaCl, 0.13M; KCl, 0.004M. Venom, 300 $\mu\text{g./ml.}$ Glucose, 0.02M. Rat-brain homogenate, 150 mg./vessel. Temperature, 37°. Gas phase, air. A, brain + glucose + CaCl_2 (0.036M); B, brain homogenate + glucose + CaCl_2 (0.002M); C, brain homogenate + glucose + heated venom + CaCl_2 (0.002M); D, brain homogenate + glucose + heated venom + CaCl_2 (0.036M).

The effects produced on the overall oxidation of various substrates by brain tissue are likely to be due to the inactivation of the pyruvic oxidase and cytochrome oxidase systems.

Closer examination of the results shows that the numerous enzyme systems not attacked by the heated venom have one common feature; they are all 'soluble' and active in aqueous tissue extracts, and their activities seem to be independent of cellular structure. Those enzymes which are inactivated by heated venom are associated with mitochondrial bodies and apparently require specific structural configuration for their activities. The succinic oxidase system is a typical example. Keilin & Hartree (1949) distinguish between two types of factors affecting this system; those reacting specifically with a catalyst in the system and

those affecting the colloidal structure of the enzyme components. Keilin & Hartree (1940) conclude that the activity of succinic oxidase is intimately associated with the integrity of the tissue particles, stating that 'efficiency of the system depends, however, not only on the integrity of its components, but also on that of the colloidal structure which supports them and assures their mutual accessibility'. Swanson & Artom (1950) have shown that a large amount of phospholipid material, present in firm combination with protein, is associated with the mitochondria. These findings, taken in conjunction with the reports that the succinic oxidase system (Hogeboom, Schneider & Pallade, 1948), pyruvic oxidase (Kennedy & Lehninger, 1948), and choline oxidase (Kensler & Langemann, 1951) are located in the mitochondria, suggest that heated venom attacks the lipoprotein complex which holds together in a particular spatial configuration the different components of these enzyme systems.

The presence of lecithinase in heated venom has been directly demonstrated by the manometric technique. By this method, it has been possible to show that heated venom can degrade, enzymically, certain structures, probably containing phospholipin, in brain tissue.

The large activity of the inhibitory factor in heated venom, which in concentrations containing 10 $\mu\text{g. protein/ml.}$ can bring about 50 % inactivation of brain pyruvic oxidase, together with the facts concerning the kinetics of the inhibition, furnish additional evidence in favour of an enzymic process being involved in the inactivation of enzyme systems by the heated venom. The accelerating action of Ca^{++} on the inactivation of glucose oxidation of brain by heated venom, coupled with the marked activating action of Ca^{++} on the lecithinase activity of venom, provides a direct link between lecithinase activity and the inhibitory effects of the venom on the various enzyme systems.

Heated venom inhibits specifically the pyruvic oxidase associated with cell structure and has no effect on pyruvic oxidase that can be extracted in cell-free aqueous solution from pigeon-breast muscle. Clearly, then, heated venom may be of direct service as a biochemical tool in its ability to discriminate between enzymes that have similar biochemical properties, but which differ from each other in their physical structure.

The observations reported here suggest that heated snake venom exerts its inhibitory effects on tissue enzyme systems by degrading, through the action of a phospholipase or phosphotidase, mitochondrial structures upon whose integrity the enzyme systems concerned depend for their optimal activities. It seems possible that venom lecithinase, by its pronounced inhibitory action on tissue

respiratory systems, as well as by its effect on acetylations (Braganca & Quastel, 1952), is concerned with some of the neurological disturbances that characterize the toxic action of snake venoms in the animal.

SUMMARY

1. Investigations have been made on the effects of snake venoms heated at 100° for 15 min. on a variety of enzyme systems. Such venoms still retain toxicity to animals.

2. The enzymic systems concerned with the oxidation of glucose, pyruvate, L-glutamate, succinate, α -ketoglutarate and fructose by brain homogenates are inhibited by small quantities of the heated venoms.

3. Pyruvic dehydrogenase of brain, succinic dehydrogenase of brain and heart, cytochrome oxidase of brain, and choline oxidase of liver are all vigorously attacked.

4. The following systems are not attacked: anaerobic glycolysis by rat brain, glucose fermentation by yeast extract, pyruvic acid dismutation by pigeon liver, hexokinase, glucose oxidase of liver, and glucose oxidase of fungi (notatin), soluble

pyruvic dehydrogenase of pigeon-breast muscle, lactic and malic dehydrogenases, and urease. Cytochrome *c* is not broken down.

5. The enzyme systems that are not attacked by heated venom are distinguished from those that are attacked by being active in aqueous cell-free tissue extracts and are apparently independent of cellular structures for their activities.

6. Heated snake venom contains a heat-stable enzyme (probably a lecithinase) that attacks brain phospholipins or lipoproteins. Its inhibiting effect on a susceptible enzyme system is enhanced by calcium ions which activate lecithinase.

7. It is suggested that heated venoms exert their inhibitive effects by enzymic degradation of mitochondrial structures upon whose integrity or particular spatial configurations the enzyme systems concerned depend for their optimal activities.

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The Phytase of Wheat

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Phytase catalyses the hydrolysis of phytic acid (inositol hexaphosphoric acid) to inositol and free orthophosphate. It was one of the first enzymes which liberate inorganic phosphate from organic phosphorus compounds to be described (Suzuki, Yoshimura & Takaishi, 1907), and it has a wide distribution in plant and animal tissues, in many species of fungi and in certain bacteria. It has not, however, been so intensively studied as many of the other phosphatases, although in recent years Courtois and his collaborators have carried out extensive work on its occurrence, specificity and mechanism of action (cf. Fleury & Courtois, 1937, 1947; Courtois, 1945, 1947; Courtois & Biget, 1943; Courtois & Joseph, 1947; Courtois & Plumel, 1947; Courtois & Perez, 1948).

In cereal grains a considerable proportion of the total phosphorus is present as phytate; in wheat, for example, the phytin P constitutes 70–75% of the total P of the grain (Booth, Carter, Jones & Moran, 1941). This is probably the source of the suggestion, frequently made, that a principal function of phytase in grains is to provide inorganic phosphate from phytate in the initial stages of germination. In this connexion Bernardini & Morelli (1912), Albaum & Umbreit (1943), de Turk, Holbert & Howk (1933) and Giri & Sreenivasan (1938) have shown that the proportion of inorganic phosphate to phytate increases progressively during germination with wheat, oats, maize and rice respectively. It has also been observed that the phytase activity of grains increases on germination, in barley by Luers & Malsch (1929), in wheat by Bernardini & Morelli (1912) and in oats by Albaum & Umbreit (1943). However, Essery (1951), although observing an increased phytase activity when barley grains germinate, found that the concomitant decrease of phytate P is relatively small and he doubts the validity of the simplified conception of phytase function mentioned above.

The relatively high content of phytate in cereals is a factor of importance in human and animal nutrition, particularly where whole grain or high-extraction products are concerned (the ratio of phytate P to total P increases markedly in the outer layers of the grain). This is because phytate can interfere with the assimilation of calcium and iron, and possibly other metal ions, due to these metals forming phytates which are highly insoluble over a wide pH range. McCance & Widdowson (1935) observed that human subjects on a high-phytate, low-calcium diet develop a negative calcium balance, and this observation provided the basis for the present practice of adding calcium carbonate to high-extraction flours. If phytate is hydrolysed by phytase then the immobilization of calcium will be decreased, and the interesting observation of Walker, Irving & Fox (1948), that in some subjects on high-phytate, low-calcium diets there is a gradual adaptation so that calcium balance is eventually restored, may perhaps be explained by an increased phytase activity of the intestinal flora.

It is useful, for both nutritional and practical reasons, to know how phytase is distributed among the different anatomical parts of the wheat grain and on its thermal stability in wheat and wheat products. No detailed work on these questions appears to have been published, and they have therefore been examined as part of a general study of wheat phytase which is described in this paper.

EXPERIMENTAL

MATERIALS

Sodium phytate. Two products have been obtained from Ciba Ltd., 'phytin' and 'sodium phytinate'. The phytin (mixed calcium and magnesium salt of phytic acid), gives, on analysis, the correct phosphorus content and the inorganic phosphate content is about 1% of the total P. If phytin is used as the enzyme substrate difficulties arise from the