The authors wish to thank Dr H. Phillips for his interest, and the Council of the British Leather Manufacturers' Research Association for their permission to publish this paper. *Acacia catechu* heartwood and harvested leaves of *Uncaria gambir* were obtained through the good offices of the State Silviculturist, Ootacamund Post, The Nilgiris, Madras State, India. We are also indebted to Dr E. C. Bate-Smith, Low Temperature Research Station, Cambridge, for a specimen of fisetin, to Professor D. Keilin, F.R.S., The Molteno Institute of Biology and Parasitology, Cambridge, for purified horse-radish peroxidase, and to Mr W. W. Reid, F.R.I.C., of Carreras Ltd., London, N.W. 1, for the acetone-dried powder of *Nicotiana tabacum*.

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The Characteristics of Hexokinase from *Locusta migratoria* Muscle

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(Received 5 February 1957)

Hexokinases from different tissues vary in their properties and differ in particular in specificity towards their sugar substrates. Those enzymes which phosphorylate a number of sugars, including both glucose and fructose, have been described conveniently by Colowick (1951) as 'non-specific', whilst Medina & Sols (1956) suggest that the term ketokinase should be used for those which attack more than one ketose, but not aldoses, and that the name fructokinase be reserved for enzymes which act only with fructose. Non-specific hexokinases have been shown to be present in yeast (Berger, Slein, Colowick & Cori, 1946; Kunitz & McDonald,

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1946), in higher plants (Saltman, 1953) and in a number of animal tissues (e.g. brain, Slein, Cori & Cori, 1950; retina, Hoare & Kerly, 1954; intestine, Sols, 1956). The specificity of the enzyme in heart muscle is similar to that of the enzyme in brain (Crane & Sols, 1955), but more than one enzyme may occur in mammalian skeletal muscle. Crane & Sols (1955) describe preparations of the non-specific enzyme from rat and dog muscle and Meyerhof (1927), in his early experiments on rabbit-muscle extracts, found that lactic acid was produced from glucose, mannose and fructose. Slein *et al.* (1950) showed that similar crude extracts could be fractionated to yield a partially purified enzyme which phosphorylated fructose but had little or no

activity towards glucose. This enzyme, like the ketokinase from liver, forms fructose 1-phosphate (Leuthardt & Testa, 1951; Hers, 1953). The occurrence of a hexokinase in insect tissue has been demonstrated by Chefurka (1954), who used homogenates of whole house-flies, and the presence in locust muscle of an enzyme which phosphorylates glucose can be inferred from the work of Rees (1954). The experiments described in this report provide evidence that the hexokinase in locust muscle is of the non-specific type and is not associated with a ketokinase.

EXPERIMENTAL

Materials. Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). It was further purified (after decomposition of the mercury salt) by passage of the cold solution through a column of Amberlite IR 100 in the acid form. The sugars used (commercial preparations, AnalaR when available) were not further purified except for glucosamine hydrochloride, which was recrystallized twice from methanol-water (1:1). Glucose and fructose 6-phosphates (G6-P and F6-P) and inosine triphosphate (ITP) were prepared in this Department and were presented to us by Dr Datta and by Dr Hoare respectively. Glass-distilled water was used for the preparation of all solutions.

Preparation of muscle extracts. Adult male *Locusta migratoria* from the Anti-Locust Research Centre, London, were fed freshly cut grass but were starved for 72 hr. before use, as preliminary experiments (see below) showed this to be advisable. Immediately before dissection the locusts were inactivated by cooling to 5° for a short time. Since it is difficult to dissect out locust muscle in quantity a procedure based on that of Rees (1954) was adopted. The head, abdomen and appendages were removed with scissors and the thoraces cut down the mid-dorsal line; after removal of the intestine and most of the salivary gland, the preparations were placed in ice-cold 0.9% (w/v) NaCl soln. until a sufficient number (up to 48) had been dissected. Each thorax was blotted and after removal of the remaining traces of salivary gland placed on a chilled watch glass and finely chopped with scissors. The mass of tissue was weighed and ground vigorously for 4 min. in a glass mortar, with gradual addition of 5 vol. of water. After adjustment of the pH to 7.5 (Johnson's short-range indicator paper) with 2N-NaOH soln. the extract was squeezed through muslin into a chilled centrifuge tube. The supernatant formed after spinning at 0° for 30 min. at 18000 g, described as the extract, was used as the source of hexokinase. The protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In preliminary tests, instead of being ground in a mortar the thoracic preparations were homogenized, either in water or in 0.2M-sucrose soln., and centrifuged at 600 g; the supernatant from such preparations is described as a homogenate.

Hexokinase assay. Muscle extracts were incubated with ATP and the appropriate sugar substrate, and the sugar remaining at the end of the incubation period was measured. Aldoses were determined by Somogyi's method (1952) and fructose by that of Bacon & Bell (1948).

The constituents of the medium shown below were pipetted into centrifuge tubes in a bath at 30°. At zero time

0.4 ml. of extract, pre-warmed to 30°, was added to each tube and after 15 min. the reaction was stopped by addition of 2 ml. each of 0.3M-ZnSO₄ soln. and 0.3N-Ba(OH)₂ soln. and 5 ml. of water. The tubes were centrifuged and sugar was estimated in the supernatant solution. Control tubes contained all solutions except ATP. The final concentration (mM) of the incubation mixture was: veronal buffer (pH 7.9), 10; hexose, 2.77; MgCl₂, 5; ATP, 5; total volume 1 ml.

In preliminary experiments in which homogenates were used NaF was also added to the incubation mixture; the volume of the mixture before addition of homogenate was then 0.5 and 0.1 ml. of 0.5M-NaF soln. was added 30 sec. after addition of the homogenate. Conditions were otherwise similar to those used for hexokinase assay in extracts.

Identification of reducing substances. In preliminary experiments (see below) when fed animals were used, reducing substances which were formed during incubation were identified chromatographically. Extracts were incubated with veronal buffer, pH 7.9, and 5 mM-MgCl₂ soln. for 2 hr., either alone or with addition of glycogen (1.67 mg./ml.); the reaction was stopped and the mixture deproteinized by addition of cold perchloric acid to give a final concentration of 5% (w/v). After neutralization with KOH soln. the precipitate was filtered off and the filtrate passed through a small column of Biodeminrolit resin (The Permutit Co. Ltd., London, W. 4) for removal of anions and cations and then freeze-dried. The residue was dissolved in a small volume of water, chromatographed on Whatman no. 1 paper by downward displacement with either *n*-butanol-acetic acid-water (4:1:5, v/v) or *n*-propanol-ethyl acetate-water (7:1:2, v/v), and developed by the benzidine reagent of Bacon & Edelman (1951).

RESULTS

Preliminary experiments

In early experiments with insects which had not been starved the reducing value of the mixture often, but not invariably, increased during incubation, making estimation of hexokinase activity by determination of the amount of sugar removed impossible. If glycogen were added as well as glucose and ATP, additional reducing substances were always formed, which suggested that they were produced by hydrolysis of glycogen present in variable amount in the extracts. When the extracts were incubated without addition of glucose or ATP and the mixture was analysed chromatographically, glucose, maltose and reducing oligosaccharides were identified whether or not glycogen had been added before incubation, indicating that amylase was present in the extracts together with sufficient glycogen to produce enough reducing products for chromatographic detection, although not always sufficient for estimation by chemical analysis. Extracts prepared from locusts which had been starved for 72 hr. never produced reducing substances detectable by the Somogyi estimation when incubated without added glycogen, and the starvation procedure was adopted as a routine.

Table 1. *Hexokinase and amylase activity of homogenates*

Homogenate (0.4 ml.) was incubated for 15 min. at 30° in the media described in the text with glucose or fructose (for detection of hexokinase activity) or with glycogen (for detection of amylase activity).

Tissue	Protein content (mg./ml.)	Hexokinase activity (μmoles of sugar utilized)		Amylase activity (appearance of reducing products)
		Glucose	Fructose	
Thoracic muscle	5.3	2.72	1.39	+
Salivary gland	10.1	0.97	0.39	+
Exoskeleton	2.1	0.08	0	-

Table 2. *Comparison of hexokinase activity of homogenate and extract*

Homogenate is the supernatant after centrifuging at 600 g and extract is supernatant after centrifuging homogenate for 30 min. at 18 000 g. Homogenate or extract (0.4 ml.) was incubated with the medium described in the text for 15 min. at 30° with or without NaF.

	Protein content (mg./ml.)	Hexokinase activity (μmoles of glucose utilized)
Homogenate with 0.05M-NaF	3.1	1.84
Extract with 0.05M-NaF	1.8	1.80
Extract without NaF	1.8	2.03

Extracts prepared by Rees's (1954) method contained exoskeleton and salivary gland in addition to muscle; therefore the hexokinase and amylolytic activity of homogenates of exoskeleton, salivary gland and carefully dissected muscle were compared (Table 1). No significant activity was detected in the homogenate from the exoskeleton but that from the salivary gland showed appreciable hexokinase and amylolytic activity, though much less, especially if the protein content of the two homogenates is taken into account, than that of the muscle homogenate. In later experiments care was taken in preparation of extracts to remove all traces of salivary gland.

Comparison of the activity of homogenates and extracts from the same preparations (Table 2) showed that, unlike the calf-brain enzyme studied by Crane & Sols (1953), the locust hexokinase was not sedimented by centrifuging at 18 000 g. It was considered preferable to use extracts rather than homogenates since centrifuging at high speed removed nearly half the protein present in the homogenate and because experiment showed that it was not necessary to add fluoride when estimating hexokinase activity in extracts (see Table 2).

Activity was not diminished by incubating extracts for 1 hr. at 30°, storing for 24 hr. at 5°, for 3 months at -10° or by freeze-drying. Dialysis for 5 hr. reduced activity somewhat and after dialysis for 18 hr. extracts were completely inactive. About half the original activity could be restored to extracts dialysed for 18 hr. by addition of Mg²⁺ or

Mn²⁺ ions; Co²⁺ ions were less effective (Table 3) and Ca²⁺, Zn²⁺, Cu²⁺ and Be²⁺ ions were without effect. Recent studies (e.g. Hers, 1952) have shown that the phosphate donor for hexokinase may be a 1:1 complex of Mg²⁺ ions and ATP, since activity is greatest when these compounds are present in the

Table 3. *Effect of addition of metal ions on the hexokinase activity of extract dialysed for 18 hr.*

Extract (0.4 ml.) was incubated for 30 min. at 30°, with the standard medium from which Mg²⁺ ions were omitted; metal ions were substituted to give a concentration of 5 mM.

Added ion	Hexokinase activity (μmoles of glucose utilized)
None	0
Mg ²⁺	1.76
Mn ²⁺	1.31
Co ²⁺	0.35

Table 4. *Effect of concentration of adenosine triphosphate and of Mg²⁺ ions on the hexokinase activity of extract dialysed for 18 hr.*

Extract (0.4 ml.) was incubated for 30 min. at 30°; concentrations of ATP and of Mg²⁺ ions were as shown. The medium contained 10 mM-veronal buffer, pH 7.9, and 2.77 mM-glucose.

Concn. of ATP (mM)	Hexokinase activity (μmoles of glucose utilized)	
	5 mM-Mg ²⁺	10 mM-Mg ²⁺
2.5	0.88	0.89
5.0	1.31	1.31
7.5	1.02	1.34
10.0	0.97	1.44
20.0	0.57	1.16

Table 5. *Effect of change of pH on the hexokinase activity of extract*

Extract (0.4 ml.) was incubated for 15 min. at 30° with the medium described in the text, except that the pH of veronal buffer was varied as shown.

pH	Hexokinase activity (μmoles of glucose utilized)
4.9	0.71
5.8	0.99
7.0	1.06
7.9	1.32
8.6	1.39

Table 6. *Relative rate of utilization of hexoses by different extracts*

Extract (0.4 ml.) was incubated for 15 min. at 30° with the medium described in the text.

Extract no.	Hexokinase activity (μ moles of hexose utilized) with added			Ratio of activity glucose:fructose
	Glucose	Mannose	Fructose	
8	2.01	—	0.57	3.5
9	1.37	—	0.47	2.9
14	1.72	—	0.64	2.9
15	2.50	1.92	0.67	3.7
16	1.78	—	0.70	2.3

Fig. 1. Rate of utilization of glucose. Extract (0.4 ml.) was incubated at 30° in the medium described in the text.

incubation medium in equimolecular proportion. This was true for locust-muscle extracts (Table 4). No phosphorylation occurred if ATP were replaced by ITP. Like other hexokinases the locust enzyme was most active at and above pH 7.9 (Table 5). Attempts to purify the enzyme by precipitation with $(\text{NH}_4)_2\text{SO}_4$ or ethanol or by the isoelectric method of Colowick (1951) were unsuccessful, as activity was destroyed by these treatments.

Sugars utilized

D-Glucose, D-mannose, D-fructose and D-glucosamine were utilized by locust-muscle extracts but not D-galactose, L-sorbose, D-ribose, D-arabinose or N-acetyl-D-glucosamine. Since ATP was necessary for utilization it is assumed that phosphorylation occurred. The relative rates at which glucose and fructose were utilized varied from one extract to another (Table 6). The rate of reaction with glucose as substrate was linear at first but later decreased somewhat (Fig. 1); it was not proportional to the amount of enzyme added (Table 7) and fell off more

Table 7. *Effect of enzyme concentration on hexokinase activity*

Varying amounts of extract were incubated in the standard medium (final vol. 1 ml.) for 15 min. at 30°.

Extract added (ml.)	Hexokinase activity (μ moles of glucose utilized)
0.4	2.5
0.3	1.5
0.2	0.7
0.1	0.1

Table 8. *Effect of addition of hexoses on the utilization of fructose*

Extract (0.4 ml.) was incubated with 2.77 mm-fructose for 15 min. at 30° in the medium described in the text.

Hexose added	Concn. of hexose added (mm)	Hexokinase activity (μ moles of fructose utilized)	
		Extract A	Extract B
None		0.76	0.66
Glucose	1.39	0.20	—
	2.77	0.04	—
	5.54	0	—
Mannose	1.39	0.16	—
	2.77	0.05	—
	5.54	0	—
Galactose	2.77	0.72	—
N-Acetyl-D-glucosamine	1.0	—	0.26
	2.0	—	0.22
	3.0	—	0.15
	4.0	—	0.07
	5.0	—	0

rapidly than the concentration; a similar effect has been observed with rat-muscle extracts (Gerritsen, 1952; Liébecq, 1952).

Phosphorylation of fructose was strongly inhibited by the presence of glucose or mannose but not by that of galactose; N-acetyl-D-glucosamine, although not itself attacked by the enzyme, also inhibited phosphorylation (Table 8). Phosphorylation of both glucose and fructose was completely inhibited by addition of either G 6-P or F 6-P at

a concentration of 5 mM. In this respect the locust enzyme resembles that from brain but not that from yeast (Crane & Sols, 1953).

Like other hexokinases which catalyse phosphorylation of glucose and mannose, the locust enzyme had a high affinity for these sugars, being saturated at a concentration of 2.77 mM. In contrast the rate of fructose phosphorylation was dependent on the initial concentration (Table 9). The Michaelis constant for fructose was calculated by the method of Lineweaver & Burk (1934) from the data given in Table 9 and was found to be approx. 5.5 mM. The low affinity for fructose was not due to too low a concentration of ATP since increase in the concentration of ATP and of Mg^{2+} ions during incubation did not affect the rate of phosphorylation (Table 9, extract 20).

Phosphorylation product

The difference of affinity of the enzyme for glucose and fructose and variation in the relative rate of phosphorylation of the two sugars from extract to extract might have been due to the presence of a ketokinase. Had this been so, on analogy with the

enzyme from liver and rabbit muscle, the reaction product would have been fructose 1-phosphate (F1-P), not F6-P, the product formed by the non-specific hexokinases. The two esters can be readily distinguished by acid hydrolysis; therefore the ester formed during incubation of fructose with a homogenate dialysed for 5 hr. (which contained negligible amounts of esterified phosphate) was isolated as the barium salt by a method similar to that of Leuthardt & Testa (1951), and its rate of hydrolysis determined. The reaction was stopped and the solution deproteinized by addition of an equal volume of 10% (w/v) trichloroacetic acid. A barium salt, insoluble in acetone but soluble in water, was isolated and dissolved in N-HCl; the barium was removed with Na_2SO_4 and the free ester analysed.

The ketose content corresponded to approximately 18% of the hexose equivalent of the total reducing value, whereas the phosphate content was somewhat greater than that to be expected for a monophosphate, which suggested that the ester was a mixture of glucose and fructose phosphates possibly contaminated with some diphosphate. On hydrolysis in N-HCl at 100° phosphate was liberated at a rate to be expected from a slowly hydrolysed hexose 6-phosphate, but there was no evidence of the presence of the rapidly hydrolysed F1-P (Fig. 2).

DISCUSSION

The substrates attacked by extracts of locust muscle, D-glucose, D-mannose, D-fructose and D-glucosamine are those phosphorylated by the non-specific hexokinase found in yeast, brain and other tissues. The isolation, as a product of fructose phosphorylation, of an ester hydrolysable only slowly by acid and having a higher reducing value than that to be expected from the ketose content, indicated the formation of a mixture of G6-P and F6-P and, assuming isomerase activity to be low, is evidence against the presence of a ketokinase. Further support for the non-occurrence of a ketokinase is afforded by the finding that fructose phosphorylation is inhibited by glucose and mannose and especially by N-acetylglucosamine, which is itself not phosphorylated (see Harpur & Quastel, 1949). The variation in the relative activity of different locust-muscle preparations towards glucose and fructose cannot be regarded as evidence for the occurrence of two different enzymes, as in crude extracts too many factors can affect the relative rates of hexose phosphorylation to make this a criterion of hexokinase specificity (cf. Wiebelhaus & Lardy, 1949). Slein *et al.* (1950) consider the low affinity of ketokinase for fructose to be characteristic of this enzyme, but this seems unlikely since the Michaelis constants reported for

Table 9. *Effect of concentration of fructose on hexokinase activity*

Extract (0.4 ml.) was incubated for 15 min. at 30° in the medium described in the text.

Concn. of fructose (mM)	Hexokinase activity (μmoles of fructose utilized)			
	8	14	15	20*
Extract no. ...				
1.39	0.35	0.45	0.38	0.34
2.77	0.57	0.64	0.67	0.62
5.54	0.88	1.05	0.82	1.01
11.08	1.13	1.38	1.13	1.18
K_m (mM)	6.2	5.8	3.7	6.3
Average	5.5			

* Concn. of both ATP and Mg^{2+} ions was increased to 10 mM.

Fig. 2. Rate of hydrolysis in N-HCl of hexose phosphate isolated after incubation of extract with fructose (see text for details).

the ketokinases from different sources differ so widely (liver <0.5 mM and rabbit muscle 0.2 M; Hers, 1955). The constant calculated for the locust-muscle enzyme, 5.5 mM, is of the same order as that found for hexokinase from yeast, 1.7 mM, by Slein *et al.* (1950).

We consider that locust muscle contains a single, non-specific hexokinase and that the enzyme is similar to that found in yeast and in brain in its general characteristics. In contrast to hexokinase from brain the locust enzyme is not sedimented by centrifuging at 18 000 *g* but, like the brain enzyme and in contrast to that from yeast, it is inhibited by the products of its action, G 6-P or F 6-P.

SUMMARY

1. Hexokinase activity has been demonstrated in extracts of thorax muscle and salivary gland of *Locusta migratoria*.

2. The characteristics of the enzyme are similar to those of the hexokinase present in brain extracts.

3. In the presence of adenosine triphosphate and Mg^{2+} ions, D-glucose, D-mannose, D-fructose and D-glucosamine are utilized.

4. Utilization of all substrates is inhibited by glucose 6-phosphate or fructose 6-phosphate, and that of fructose by glucose, mannose and N-acetylglucosamine.

5. An ester stable to acid hydrolysis, probably a mixture of glucose 6-phosphate and fructose 6-phosphate, has been identified as a product of phosphorylation of fructose.

6. Extracts of muscle and of salivary gland contain an amylase which hydrolyses glycogen present in extracts of fed insects.

We should like to thank Dr S. P. Datta for the gift of hexose phosphates, Dr D. S. Hoare for inosine triphosphate and the Director of the Anti-Locust Research Centre for locusts.

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The Hexose Specificity of Hexokinase in Brain from Various Species

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(Received 5 February 1957)

Geiger (1940) was the first to demonstrate hexokinase activity in extracts of rat brain, and later workers have shown the enzyme to be present in ox (Colowick, Cori & Slein, 1947), sheep (Slein, Cori & Cori, 1950) and mouse (Hesselbach & DuBuy, 1953)

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brain. The studies of later workers [especially that of Sols & Crane (1954) on calf brain] indicate that the enzyme in mammalian brain resembles that from yeast in its ability to attack a number of sugars. The experiments described below show that an enzyme with a similar specificity is found in brain tissue of birds, fishes, amphibia and molluscs.