adenine is at least of the same type as the enzymes (or enzyme) that oxidize the above-mentioned group of substrates.

SUMMARY

1. The enzymic oxidation product of adenine by milk xanthine oxidase has been identified as 2:8-dihydroxyadenine.

2. An enzymic differential spectrophotometric method for the determination of adenine is described.

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Studies Involving Enzymic Phosphorylation

1. THE HEXOKINASE ACTIVITY OF RAT TISSUES

By C. LONG
Department of Biological Chemistry, University of Aberdeen

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Colowick & Kalckar (1941, 1943) have demonstrated that the enzyme hexokinase catalyses the transfer of the terminal phosphate group of adenosinetriphosphate (ATP) to glucose or fructose, yielding the corresponding hexose-6-phosphate and adenosinediphosphate (ADP). Mannose (Berger, Slein, Colowick & Cori, 1946) and glucosamine (Harpur & Quastel, 1949) have also been found to serve as substrates. Mg^{++} is required as a cofactor in these phosphorylations.

The literature concerning the hexokinase activity of animal tissues is extremely fragmentary. Values for homogenates of rat brain and liver have been reported by Utter (1950) and by Vestling, Mylroie, Irish & Grant (1950), respectively. The published figures for rat kidney (Stadie & Haugaard, 1949) and skeletal muscle (Colowick, Cori & Slein, 1947; Stadie & Haugaard, 1949), however, refer to extracts only and were obtained in attempts to discover whether or not the hexokinase activity of animal tissues could be influenced by the *in vitro* addition of hormonal extracts; the lack of agreement in this latter field could conceivably have been due to variations in the experimental conditions adopted by the different workers. Partly in view of this possibility and partly because of the intrinsic value of such information, it seemed desirable to establish the optimal conditions for determining the hexokinase activity of animal tissues and to measure quantitatively the maximal glucose-phosphorylating capacity of each tissue.

In the work to be described, rat-tissue homogenates have been studied and the requirements for maximal activity have been found to be remarkably similar in all tissues. Brain was found to exhibit the greatest hexokinase activity while liver was least active. Other tissues varied over a fourfold intermediate range of activity.

A secondary aim of the present work was to ascertain whether any obvious correlation existed between the hexokinase activity of a tissue and its known function in the economy of the whole animal. As a result of the many enzyme assays performed, no close correspondence could be found for kidney or intestine, in spite of the fact that the phosphorylation of carbohydrate is generally regarded as occupying a special place in the metabolism of these tissues.

EXPERIMENTAL

Materials

Adenosinetriphosphate. ATP was prepared from rabbit muscle as the dibarium salt ($C_{10}H_{12}O_{13}N_5P_3Ba_2.4H_2O$) according to the procedure of Dounce, Rothstein, Beyer, Meier & Freer (1948). Analysis of freshly prepared material: total P, 10·4% (calc. 10·9%); P hydrolysable by N-H_2SO_4 in 10 min. at 100°, 7·3% (calc. 7·3%); hydrolysable P as fraction of total P, 0·70 (calc. 0·67); total N (Kjeldahl),

8·2% (calc. 8·2%); the product was completely free from inorganic orthophosphate. It was stored at −20°; Ba was removed by means of a slight excess of K_2SO_4 to give a 0·05M-stock solution of potassium ATP, which was kept in the refrigerator and freshly prepared about once each month.

Other compounds used were of analytical reagent grade or best available quality and were obtained commercially. Media for the enzymic incubations were prepared in glass-distilled water.

Animals. Hooded rats of the Rowett Institute strain have been used throughout. They were mostly taken from the stock colony maintained by the Physiology Department of this University, but a few were obtained directly from the Rowett Institute, Bucksburn, Aberdeenshire; from the time of weaning they had been fed *ad lib.* a stock diet of rat cake (purchased from the North-Eastern Agricultural Co-operative Society Ltd.). The rats were not fasted except when liver was being assayed; in these cases the animals were deprived of food but not water for 43 hr. in order to reduce the liver glycogen content. Fasting was found to have no effect on the hexokinase activity of other tissues. Male rats, about 3 months old, average weight 300 g., were used, female rats being employed only when uterus was one of the tissues being studied. As there were no apparent sex differences between the hexokinase activities of other tissues, the results obtained on male and female rats have been pooled.

Methods

Animal tissues. The rats were killed by decapitation and exsanguination; the tissues were immediately dissected out and immersed in ice water. After about 2 min., the excess fluid was removed by blotting lightly with filter paper and a portion of cold moist tissue was weighed and homogenized in ice-cold 0·12M-potassium phosphate buffer, pH 7·8, containing 0·15M-KF (5 ml./g. tissue) so that the homogenate contained 0·10M-phosphate and 0·125M-fluoride.

With stomach, small intestine, caecum or colon the organ was dissected free from the mesentery, slit longitudinally and washed in a large beaker in a stream of running tap water to remove undigested food residues and other unwanted material without detaching the mucosa. The tissue was then blotted between filter papers and weighed. When the mucosa of the small intestine was wanted the clean, blotted intestine was placed on a glass plate and the mucosa scraped away from the muscular layers using a blunt spatula. About 45–50% of the weight of the small intestine was removed in this way and this fraction has been designated 'mucosa'.

Dry weights. After drying overnight at 105°, the following percentage dry weights were found for the rat tissues studied: brain, 22·9; caecum, 27·2; colon, 18·3; heart, 22·4; small intestine, 20·8; kidney, 22·9; liver, 31·4; lung, 18·4; skeletal muscle, 22·2; pancreas, 32·4; spleen, 23·8; stomach, 24·0; testis, 12·5; uterus, 19·1.

Preparation of homogenates. In addition to an all-glass homogenizer of the Potter & Elvehjem (1936) type, two other methods have been used. With liver, brain, kidney, heart, testis, uterus, pancreas and intestinal mucosa, suitable homogenates could readily be prepared simply by grinding the tissue in an ice-cold mortar with gradual addition of medium; indeed, for these particular tissues this was the method of choice. In every case it was found that similar hexokinase activities were obtained when these two methods were compared, using tissue from the same animal. Whole small intestine and intestinal muscle, were usually homogenized in an apparatus of the type described by Folley & Watson (1948); this technique also gave values for hexokinase activity similar to those obtained with a Potter & Elvehjem homogenizer. The homogenates were not filtered through gauze for they could readily be pipetted by means of an ordinary 1 ml. graduated pipette with an enlarged tip.

The enzymic phosphorylation of glucose. Solutions containing appropriate amounts of glucose, potassium ATP, $MgCl_2$ and KCl were pipetted into 15 ml. centrifuge tubes, made up to 0·3 ml. with glass-distilled water, and immersed in a Warburg bath at 30°. The homogenate in phosphate-fluoride medium (0·2 ml., containing 33 mg. fresh tissue), prewarmed to 30°, was pipetted in at zero time and the contents gently mixed. After incubation, usually not longer than 10 min., 0·5 ml. 0·3N-$Ba(OH)_2$ was added in order to arrest enzyme activity. After removal from the bath, 5% (w/v) $ZnSO_4$ (0·5 ml.) was added to precipitate the proteins and adsorb the glucose-6-phosphate and other phosphate esters (Somogyi, 1945). Exactly 3·5 ml. water was then added and the contents of the tube thoroughly shaken, centrifuged for about 5 min. at 2000 rev./min. and the almost clear supernatant filtered through a 7 cm. Whatman no. 42 filter paper. 1 ml. of the filtrate was taken for glucose determination according to Nelson (1944), the final colour comparisons being made in a photoelectric colorimeter (Evans Electroselenium Ltd.). While the incubation was proceeding, 0·3 ml. of a standard glucose solution and a similar volume of water were separately treated first with 0·3N-$Ba(OH)_2$ (0·5 ml.) and then with homogenate (0·2 ml.) followed by 5% $ZnSO_4$ (0·5 ml.) and carried through the same procedure except that the incubation was omitted. The amounts of glucose present in the various tubes were calculated from their colorimeter readings together with those of the standard glucose and blank.

RESULTS

Determination of optimal conditions for glucose phosphorylation

General considerations. Glucose was chosen as substrate rather than fructose on account of its much greater affinity for the enzyme, the Michaelis constants for glucose and fructose being $1·45 \times 10^{-4}$ and $1·3 \times 10^{-3}$M, respectively (Wiebelhaus & Lardy, 1949; Slein, Cori & Cori, 1950). Moreover, certain tissues, such as liver and muscle, contain both hexokinase and a specific fructokinase (Slein *et al.* 1950; Vestling *et al.* 1950); thus, results obtained with fructose would be due to the combined effect of both enzymes in these tissues. Although mannose (Michaelis constant, $1·0 \times 10^{-4}$M; Slein *et al.* 1950) is somewhat superior to glucose in its affinity for hexokinase, the maximal rate of mannose phosphorylation is only about 30–40% of that found for glucose (Berger *et al.* 1946).

Earlier workers have usually determined hexokinase activity either by following manometrically the rate of formation of acid groups or by measuring the rate of disappearance of easily hydrolysable (10 min. in N acid at 100°) P. With crude tissue homogenates, however, these methods are certain to give erroneously high values, because the activities of phosphofructokinase, adenosinetriphosphatase and other phosphatases will contribute to the

formation of acid groups, while the first two enzymes will also cause increased loss of easily hydrolysable P. The rate of glucose disappearance has therefore been employed for the determination of hexokinase activity. Broh-Kahn & Mirsky (1948) have argued along similar lines.

Homogenates have been preferred to extracts in this study, for it has been found that a variable fraction of the hexokinase activity of a homogenate passes into the extract. Thus, while 100% of the activity of a stomach homogenate was present in the supernatant after centrifuging at 9000 *g* (angle centrifuge), in two experiments with kidney the corresponding figures were only 14 and 54%; intestinal mucosa gave a value of 57%. Hence, the activity of an extract depends on the nature of the tissue studied and probably also on the thoroughness of centrifugation. In preliminary experiments, the enzymic reaction was carried out under N_2 or a 95% N_2–5% CO_2 mixture, but similar rates of glucose utilization were observed when the incubations were conducted in air. It was also found to be unnecessary to shake the tubes during the incubation.

In the standard test system, 0·2 ml. homogenate, equivalent to 33 mg. wet weight of tissue, was routinely used in a reaction mixture of total volume 0·5 ml. at 30° with a medium of the following final composition, which was found to be optimal: glucose (0·0012 or 0·0024 M), potassium ATP (0·005 M), $MgCl_2$ (0·005 M), KF (0·05 M), potassium phosphate buffer, pH 7·8 (0·04 M) and KCl (0·042 M). Control experiments were always carried out using the complete system without ATP.

Temperature. A few additional experiments were carried out at higher temperatures. The Q_{10} values obtained for homogenates of rat brain, kidney and intestinal mucosa in the range 30–40° were 1·60, 1·47 and 1·52, respectively (average, 1·53).

Cation composition of the medium. Wiebelhaus & Lardy (1949) have shown that the hexokinase activity of an ox-brain extract is somewhat inhibited by an excess of Na^+. In an experiment using rat intestinal mucosa homogenate, it has been confirmed that Na^+ exerts an inhibitory effect on the rate of glucose phosphorylation which is just detectable (Table 1). In view of this finding, an all potassium medium has been used in all subsequent experiments reported in this paper.

Table 1. *Dependence of hexokinase activity on cation composition of reaction medium*

(Rat intestinal mucosa. 0·2 ml. homogenate (≡33 mg. moist tissue) in total volume 0·5 ml. Final concentration of added glucose, 0·0024 M; concentrations of ATP, $MgCl_2$, KF and potassium phosphate, pH 7·8, as for standard test system. Amounts of NaCl and KCl varied to give final concentrations of Na^+ and K^+ as shown. 4 min. incubation at 30°.)

Final concn. (M)		Glucose utilized
Na^+	K^+	(μg.)
0·022	0·165	69
0·043	0·144	69
0·064	0·123	61

Effect of pH *value of the medium.* Three types of experiment have been carried out to test the effect of pH variations on the hexokinase activity: (*a*) tissue homogenates prepared in unbuffered 0·125 M-KF (final concentration) were pipetted into reaction media containing potassium phosphate buffer at various pH values together with all other components of the complete system. (*b*) Weighed portions of tissue were separately homogenized in phosphate-fluoride media of different pH value and the resulting homogenates were then added to the other components of the complete system. (*c*) Tissue homogenates prepared in a phosphate-fluoride medium of pH 7·8 were added to tubes containing the other components of the test system which had earlier been treated with accurately measured amounts of 0·025 N-KOH or 0·125 N-HCl to give known final pH values. In all cases pH values were determined on reaction mixtures containing homogenate, using a pH meter with glass electrode.

The results of these experiments are shown in Table 2. While the hexokinase activity of tissue homogenates did not appear to be highly sensitive to pH changes, optimum values were usually obtained in the pH range, 7·5–8·0. In view of these findings, homogenates have been prepared in phosphate buffer, pH 7·8, for routine determinations of hexokinase activity.

Table 2. *Hexokinase activity of rat-tissue homogenates at varying* pH *values*

(Methods (*a*), (*b*) and (*c*) described in text. Final concentrations of ATP, $MgCl_2$, KCl, KF and potassium phosphate as for standard test system. In Exp. 3, final glucose concentration, 0·0024 M; 10 min. duration. In all other experiments, final glucose concentration, 0·0012 M; 8 min. duration.)

Exp. no.	Method	Rat tissue	Final pH value	Glucose utilized (μg.)
1	(*a*)	Kidney	6·20	56
			6·60	52
			7·09	94
			7·57	117
			7·90	115
			8·15	104
2	(*a*)	Intestinal mucosa	6·38	107
			6·81	107
			7·31	108
			7·80	134
			8·15	95
			8·56	89
3	(*b*)	Intestinal mucosa	6·38	88
			7·80	92
			8·56	72
4	(*b*)	Kidney	7·80	56
			8·55	57
5	(*c*)	Kidney	5·71	59
			6·38	64
			7·49	54
			7·87	55
			8·19	58

Effect of glucose concentration. In rat intestinal mucosa and stomach homogenates the rate of glucose utilization hardly varies with glucose concentration over the range studied. This effect is shown in Table 3. In their study of the hexokinase activity of ox-brain extracts, Wiebelhaus & Lardy (1949) experienced a similar behaviour, for on raising the glucose concentration from 0·0005 to 0·005 M, the rate of phosphorylation was not increased by more than 5%.

Table 3. *Hexokinase activity at varying glucose concentrations*

(Standard test system, except for varying glucose concentration.)

Initial glucose concentration (M)	Glucose utilized (μg.) Intestinal mucosa (4 min.)	Stomach (5 min.)
0·0012	74	89
0·0024	77	—
0·0036	80	97

Effect of ATP concentration. With glucose as substrate, Slein *et al.* (1950) determined the affinity of crystalline yeast hexokinase for ATP and reported a Michaelis constant of $9{\cdot}5 \times 10^{-5}$ M. With ox-brain extracts, Wiebelhaus & Lardy (1949) found that an initial ATP concentration of 0·002 M was sufficient for maximal activity when fairly small amounts of glucose were being utilized. In the present work (Table 4) a somewhat higher concentration of ATP was

Table 4. *Hexokinase activity at varying ATP concentrations*

(Standard test system, except for variations in ATP concentration.)

	Glucose utilized (μg.) Intestinal mucosa (8 min.)	Heart (6 min.)	Kidney (8 min.)
Initial glucose (M)	0·0024	0·0012	0·0012
ATP concentration (M)			
0·0000	−4	20	2
0·0013	60	48	34
0·0025	97	60	45
0·0038	118	84	66
0·0050	139	86	71
0·0063	138	—	71

required to give the maximal rate of glucose utilization, presumably because of the competitive destruction of ATP by crude homogenates (Meyerhof & Geliazkowa, 1947). Maximal hexokinase activity was always obtained when the concentration of ATP reached 0·005 M. In most tissues there was a slight formation of reducing substance, estimated as glucose, during the course of the incubation in the absence of ATP. Where this effect was observed, the glucose utilization has been taken to be the difference between the amounts of glucose present in the complete system and in the same without ATP at the end of the incubation period. An effect of similar nature has also been noted by Stadie, Haugaard & Hills (1950) using an extract of cat skeletal muscle.

Effect of Mg^{++} *concentration.* Under conditions which are otherwise optimal, but without added Mg^{++}, the rate of glucose utilization by tissue homogenates was very low; maximal activity appeared to be obtained when the concentration of added $MgCl_2$ reached 0·005 M (Table 5).

The actual concentration of Mg^{++} in these unsupplemented tissue homogenates has not been determined. However, Leut'skii (1946) has reported normal values of 101, 69 and 78 mg. Mg/100 g. for rat muscle, brain and liver, respectively. Assuming an average value of 80 mg./100 g. tissue, the unsupplemented reaction mixtures, as prepared in the present work, should contain about 0·002 M-Mg^{++}. Thus it is quite likely that Mg^{++} is essential for hexokinase activity.

Table 5. *Effect of* Mg^{++} *concentration on hexokinase activity*

(Standard test system, except for Mg^{++} concentration.)

	Glucose utilized (μg.) Intestinal mucosa (8 min.)	Brain (5 min.)	Testis (8 min.)
Initial glucose (M)	0·0024	0·0024	0·0012
Mg^{++} concentration (M)			
0·0000	48	38	23
0·0015	92	107	63
0·0030	103	116	78
0·0050	110	127	84
0·0075	101	135	75

Effect of fluoride concentration. In most animal tissue preparations, the hydrolysis of glucose-6-phosphate at pH 7·8 is probably brought about by non-specific alkaline phosphatase. In liver, kidney and possibly intestinal mucosa, a specific glucose-6-phosphatase is also active (Broh-Kahn, Mirsky, Perisutti & Brand, 1948; Swanson, 1950; Duve, Berthet, Hers & Dupret, 1950; Hers & Duve, 1950). In general, fluoride ions are known to inhibit phosphate-splitting enzymes, and in the present work the rate of disappearance of glucose from the reaction mixture has often been found to be raised appreciably by increasing concentrations of KF (Table 6); where an effect is seen, it appears to be maximal at about 0·05 M-KF. The presence of fluoride in the medium has the additional advantage of inhibiting ATPase to a considerable extent.

Table 6. *Effect of fluoride concentration on hexokinase activity*

(Standard test system, except for variable KF concentration.)

	Glucose utilized (μg.) Kidney (8 min.)	Pancreas (6·5 min.)	Intestinal mucosa (10 min.)
Initial glucose (M)	0·0012	0·0012	0·0024
KF concentration (M)			
0·000	30	38	72
0·015	60	37	114
0·030	66	37	114
0·050	71	35	112
0·075	57	40	107

In the case of kidney (Table 6), the need for fluoride is evident and has consistently been found. In pancreas, however, fluoride has no noticeable effect and a similar result has been obtained for lung. With intestinal mucosa the response was variable; in the experiment shown (Table 6), maximal hexokinase activity was seen in the presence of 0·015 M-KF, higher concentrations producing no further activation. In another experiment (not quoted) fluoride was without effect. This latter result is surprising, especially in view of the finding by Hers & Duve (1950) that glucose-6-phosphate is optimally hydrolysed by intestinal mucosa at pH 8·0.

That the fluoride ion, up to a concentration of 0·1 M, has no inhibitory effect on hexokinase has been shown for the crystalline yeast enzyme by Berger *et al.* (1946). A similar result is that shown for pancreatic tissue (Table 6), the hexokinase activity of which is not inhibited by KF up to 0·075 M. In view of the hydrolysis of glucose-6-phosphate which would normally take place in homogenates of tissues such as kidney, routine determinations of hexokinase activity have been carried out in the presence of 0·05 M-KF. In order to avoid the precipitation caused by the interaction of fluoride, ATP and Mg^{++} in concentrated solution, the tissue was homogenized in a fluoride-phosphate medium and added to the rest of the reaction mixture containing ATP and Mg^{++}. In experiments on guinea pig brain, Case & McIlwain (1951) have recommended that the homogenization of the tissue be carried out in a fluoride-containing medium in order to reduce the phosphatase activity.

Since any method of hexokinase assay, based on the rate of disappearance of glucose, requires that enzymic hydrolysis of the phosphorylated product, glucose-6-phosphate, should not take place, separate experiments have been carried out to determine whether 0·05 M-KF would quantitatively inhibit the hydrolysis of added glucose-6-phosphate. It has been found in the case of homogenates of every tissue studied, that this fluoride concentration completely prevented the formation of reducing substances from hexosemonophosphate (equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate) at an initial concentration of 0·0005 M, i.e. at a concentration equivalent to that produced by phosphorylation of more than 80% of the glucose used in the standard test system.

Effect of enzyme concentration. In those tissues which were examined, the rate of glucose utilization was directly proportional to the volume of homogenate taken, within the limits of experimental error, other components of the medium being maintained constant (Table 7). The absence of any falling off in the rate of reaction either at lower or higher concentrations of homogenate may be taken as evidence that there are no other co-factors to be considered and that the known co-factors are present in optimal amount.

Table 7. *Dependence of rate of glucose utilization on volume of homogenate taken*

(Standard test system, except for variation in amount of tissue taken. 1 ml. homogenate equivalent to 167 mg. fresh tissue.)

	Glucose utilization (μg.)		
	Testis (10 min.)	Brain (6 min.)	Intestinal mucosa (7 min.)
Initial glucose (M)	0·0012	0·0024	0·0012
Homogenate (ml.)			
0·05	19	38	27
0·10	43	73	52
0·20	72	167	88

Effect of duration of incubation. Provided not more than about 80% of the initial glucose was used up, the following rat tissues showed strict proportionality between duration of incubation and extent of glucose utilization: brain, caecum, colon, heart, small intestine, kidney, lung, stomach, testis and uterus; i.e. the reaction was of zero order. In the case of intestinal mucosa, the reaction rate decreased appreciably as the time of incubation was lengthened; in twenty-two experiments, the average quantity of glucose disappearing during a second 4 min. period was only 72% of the amount utilized during the first 4 min. Thus in calculating the hexokinase activity of intestinal mucosa, only the early period of incubation has been considered. With pancreas, a similar but even more pronounced effect was observed and in this case too, only data referring to short periods of incubation have been employed.

With rat skeletal muscle, an opposite effect was seen. During short incubation periods of about 5 min. or less, a negligible amount of glucose was utilized; indeed, the presence of ATP in the medium often appeared to increase the amount of glucose present, as compared with a control without ATP, so that negative rates of glucose utilization were observed under these conditions. With longer periods of incubation, however, a positive and linear rate of reaction was consistently found. For this reason, when calculating the hexokinase activity of skeletal muscle homogenates, it has been necessary to discard the earlier values and use only the figures relating to the longer periods of incubation, e.g. for the period 6–12 min. Spleen behaved in a similar manner. Perhaps the simplest explanation for these observations would be that these tissues contained a hexokinase inhibitor which gradually underwent destruction during the incubation.

Liver presented a special problem owing to its high glycogen content. Fasted rats were necessarily used when assaying this tissue for hexokinase, for otherwise the excessive formation of glucose during the preparation of the homogenate and subsequent incubation flooded the system to such an extent that it was impossible to observe any disappearance of glucose during the test period. Even with liver from fasted rats, there was still sufficient glycogen to cause a net increase in glucose concentration during the first few minutes of incubation. However, it was found that if a preliminary incubation period of 10 min. at 30° was allowed before mixing the homogenate with the other components of the system, a small though measurable amount of glucose was utilized at a rate approximately proportional to the duration of the test.

The hexokinase activity of rat tissues

A summary of the results of individual experiments is recorded in Table 8, in which the tissues

Table 8. *Hexokinase activity of rat tissues*

(Standard test system.)

Tissue	No. of exps.	$-Q_{glucose}$ Average	$-Q_{glucose}$ Range
Brain (whole)	6	27·1	20·3–31·2
Colon	4	17·2	12·6–21·4
Heart	9	14·5	9·2–17·8
Stomach	3	14·5	8·3–20·4
Testis	6	13·7	11·4–18·8
Small intestine	3	11·7	9·3–13·3
Caecum	2	10·7	10·4–10·9
Uterus	4	8·5	6·0–10·9
Spleen	3	8·3	7·3– 9·3
Kidney (whole)	12	7·9	6·3–11·0
Skeletal muscle	8	7·4	1·7–12·0
Pancreas	4	5·6	4·0– 7·4
Lung	3	4·3	2·9– 5·6
Liver	8	1·4	0·2– 2·6

have been arranged in order of decreasing hexokinase activity. The hexokinase activities of the mucosal and muscular portions of small intestine are compared in Table 9. Hexokinase activity is given by the rate of glucose utilization and has been calculated in terms of $-Q_{glucose}$ (μl. glucose disappearing/mg. dry wt. of tissue/hr.).

Table 9. *Hexokinase activities of the mucosal and muscular portions of small intestine*

(Standard test system. In each experiment, the mucosa and muscle were taken from the small intestine of the same animal.)

	$-Q_{glucose}$	
Exp. no.	Mucosa	Muscle
1	14·1	11·8
2	17·6	13·9
3	10·1	9·7
Average	13·9*	11·8

* Average of eleven experiments, 14·8 (range, 10·1–20·2).

DISCUSSION

Perhaps the most striking fact which emerges from an inspection of the data in Table 8 is the very high hexokinase activity of rat-brain homogenate, which is almost double that of any other tissue, and the very low activity of rat liver. In Table 10 the maximal phosphorylating capacities at 38° of various rat-tissue homogenates have been calculated in terms of mg. glucose/whole tissue/hr. In order to convert from 30 to 38°, a factor of 1·41 has been employed, corresponding to an average Q_{10} value of 1·53 (see p. 409).

Table 10. *Maximal glucose phosphorylating capacities of rat tissues*

(The rates of glucose phosphorylation at 38° have been calculated from the following data: (*a*) $-Q_{glucose}$ at 30° (Table 8); (*b*) average $Q_{10}=1{\cdot}53$; (*c*) moist weight of tissue; and (*d*) percentage dry weight of tissue. See Experimental section, p. 409.)

Tissue	Average wt. of whole moist tissue (g.)	Maximal rate of glucose phosphorylation (mg./whole tissue/hr.)
Brain	1·89	132
Caecum	1·76	57
Colon	1·97	70
Heart	1·00	38
Kidneys	2·27	46
Liver	6·59*	33
Lung	1·58	14
Pancreas	0·92	20
Skeletal muscle	150†	2780
Small intestine	9·12	251
Intestinal mucosa	4·37	152
Spleen	1·98	44
Stomach	1·59	62
Testes	2·28	45
Uterus	0·44	9

* Fasted liver used here; weight of non-fasted liver, approx. 10 g.

† Approximate only, assuming 50% of a 300 g. rat to be muscle.

Brain. The dependence of the *in vivo* respiratory metabolism of brain upon the level of the blood glucose would lead to the expectation of a high hexokinase activity for this tissue. Utter (1950), working with a homogenate of cotton rat brain, obtained a value of 37·8 for $-Q^{O_2}_{glucose}$ at 38°. When the temperature difference is taken into account, the average value of 27·1 at 30° found in the present work is equivalent to 38·2 at 38°, which agrees closely with Utter's (1950) figure. Such a rate of glucose phosphorylation is considerably higher than that required to support the observed rate of respiration of rat brain, for Krebs (1934) has quoted an average $-Q_{O_2}$ value of 10·7 for slices of rat-brain cortex at 38°, a figure which would require a $-Q_{glucose}$ value of only 1·8, assuming complete oxidation of the glucose molecule. Reiner (1947) has also considered this problem, and while he did not determine directly the glucose utilization of rat-brain homogenates, the manometrically determined values of $-Q_{O_2}$ and $Q^{O_2}_{glycolysis}$ indicated that for every molecule of glucose completely oxidized, 12 mol. of glucose were converted into lactic acid. Thus it appeared that the rate of pyruvate oxidation was the limiting factor in the complete oxidation of glucose by rat brain. Under *in vivo* conditions, it is possible that suboptimal concentrations of co-factors and the presence of uninhibited phosphatases would reduce the net rate of glucose phosphorylation considerably. Whatever may be the complete explanation, the results clearly show that the hexokinase activity of the brain is more than sufficient to meet the metabolic requirements of the rat.

Kidney. All previously published data on the hexokinase activity of kidney refer to extracts. Glucose phosphorylation in kidney extracts was first observed by Colowick, Welch & Cori (1940) using the ox, lamb, pig, cat and rabbit, but their results do not indicate which species was used in any given experiment. Later Youngburg (1944), using rabbit-kidney cortex extracts at 39–40°, obtained data from which a $-Q_{glucose}$ value of 4·2 may be calculated. More recently, Stadie & Haugaard (1949) have employed rat-kidney extracts at 30° under conditions rather similar to those used in the present study except for the addition of adrenal cortex extract, which the authors stated to be without effect; their results may be recalculated to give an average $-Q_{glucose}$ value of 2·4. In the present work, the determinations made upon kidney homogenate and extract, already referred to, have indicated that not more

than about 50 % of the hexokinase activity remains in the supernatant, so that the higher average $-Q_{glucose}$ value of 7·9 (Table 8) obtained for kidney homogenate is to be expected.

It seemed of interest to ascertain whether the $-Q_{glucose}$ value observed in this study could be correlated with the performance of rat kidneys under physiological conditions. No data could be found in the literature for the maximal rate of glucose re-absorption from the glomerular filtrate in the rat, but experiments on inulin clearance by Friedman & Livingstone (1942) and Dicker & Heller (1945) have yielded values of $0{\cdot}27 \pm 0{\cdot}06$ and $0{\cdot}35 \pm 0{\cdot}04$ ml./100 g. body weight/min. respectively for the glomerular filtration rates of albino rats. Taking an average figure of 0·30 ml./100 g. body weight/min. together with a mean value of 120 mg. glucose/100 ml. arterial blood found for the rats from our colony (Taylor, 1951), and assuming the glucose concentrations in whole blood and plasma to be equal, it can be calculated that a 300 g. rat must be able to reabsorb not less than 65 mg. glucose/hr. from the glomerular filtrate under normal conditions. This figure is to be compared with the value of 46 mg. which is the maximal amount of glucose which may be phosphorylated per hr. as calculated from the hexokinase activity (Table 10). The difference would be even greater at a blood glucose level corresponding to a renal threshold of about 180 mg. glucose/100 ml. blood. This discrepancy is difficult to resolve unless it be assumed that under hyperglycaemic conditions about half of the glucose of the glomerular filtrate is reabsorbed by passive diffusion. That this may be the true explanation is suggested by the observations of Hemingway (1939) who determined the ratio of the inulin and xylose clearances in the dog and found that about 31 % of the pentose of the glomerular filtrate was reabsorbed, presumably by a passive mechanism.

Intestine. The hexokinase activity of the mucosal portion of rat small intestine appears to be slightly greater than that of the muscular portion (Table 9). Apparently also the glucose phosphorylating capacity of rat colon is higher than that of small intestine (Table 8). Since it has usually been accepted that 'active' absorption of sugars does not take place from the colon, stomach or caecum, the fact that their hexokinase activities are comparable in magnitude with that of intestinal mucosa is somewhat surprising.

The recently published data of Hele (1950) on glucose phosphorylation by rat intestinal mucosa at 30°, when converted into $-Q_{glucose}$ units, yield average values of 23·4 (water homogenates) and 37·5 (magnesium Ringer homogenates). These figures are nearly double those found in the present work.

The diet of the adult hooded rats used in this research normally contains about 12 g. carbohydrate/day. Table 10, however, shows that the mucosa of the small intestine possesses a phosphorylating capacity of only 152 mg. glucose/hr. or 3·65 g./day. In fact, in order to account for the phosphorylation of 12 g. carbohydrate/day, it would be necessary to take into consideration the whole of the alimentary tract. Even if the highest hexokinase activity of Hele (1950) were employed in the calculation, the total absorption of this amount of glucose could not be accounted for by the mucosa alone. In experiments to determine the *in vivo* rate of absorption, Hele (1950) found that 57 mg. glucose were absorbed in 30 min. This would give an absorption rate of only 2·7 g. glucose/day, a value which is probably much too low, for Cori (1925) obtained an average figure of 172 mg. glucose/100 g. body weight/hr., equivalent to 12·4 g./24 hr.

As in the case of kidney, the possibility must be considered that a fraction of the glucose is absorbed from the intestine by passive diffusion.

Skeletal muscle. The hexokinase activity of homogenates is probably significantly higher than of extracts of skeletal muscle. The $-Q_{glucose}$ value of 7·4 (Table 8), found for homogenates, is to be compared with 2·8 and 2·6 calculated from the data of Colowick *et al.* (1947) and Stadie & Haugaard (1949), respectively, for extracts.

The anomalous behaviour of rat-skeletal muscle homogenates during short incubation periods has already been referred to. This initial period of inhibition superficially resembles that observed by Colowick *et al.* (1947) with muscle extracts from alloxan-diabetic rats, and in further experiments has been found to be relieved by insulin. The fact that a spleen homogenate behaved similarly may be related to the observation of Broh-Kahn & Mirsky (1947) that spleen extracts inhibited the hexokinase activity of muscle extracts in the same manner as anterior pituitary extract.

Liver. The low hexokinase activity of rat-liver homogenates was not unexpected, although Slein *et al.* (1950), by contrast, consistently observed fairly high rates of glucose phosphorylation with homogenates from the livers of their strain of rats. In a recent paper, Vestling *et al.* (1950), on the other hand, found that their rat-liver homogenates readily catalysed the phosphorylation of fructose by ATP, but had little action on glucose. This observation was not caused by rapid dephosphorylation of the glucose-6-phosphate formed by glucose-6-phosphatase for the liver homogenate had no effect on the activity of a brain-hexokinase preparation.

These latter results are also in agreement with the findings of Chernick & Chaikoff (1951) who incubated rat-liver slices in a medium containing

uniformly labelled [^{14}C]-glucose and determined the amount of ^{14}C incorporated into the fatty acids and respiratory carbon dioxide. If it be assumed that the formation of these two products accounts for the whole of the glucose utilized, then a $-Q_{glucose}$ value of only 0·8 can be calculated from their data. The possibility must be considered, however, that other reactions involving C_2 fragments, derived from [^{14}C]-glucose, are also taking place, e.g. acetylation, formation of ketone bodies, etc. A recent paper by Sacks (1951) may also be taken as support for the present findings. This author studied the relative specific activity: time relationships of the acid-soluble phosphate esters of rat liver after subcutaneous injection of inorganic ^{32}P, and presented evidence against the idea that the labile P of ATP could be the precursor of the stable P of glucose-6-phosphate.

Table 10 shows that the maximal glucose phosphorylating capacity of rat liver is only about 33 mg./hr. This value is considerably lower than many of the published figures for the rate of liver-glycogen synthesis in the intact rat. Thus Treadwell, Tidwell & Grafa (1943) on feeding glucose to rats previously fasted for 24 hr. observed an average rate of liver-glycogen synthesis of 74 mg./hr., corresponding to a glucose equivalent of 82 mg./hr.

Parallel with these observations is the well known difficulty of demonstrating the conversion of glucose into glycogen in the presence of liver slices from fasted rats or rabbits (Cross & Holmes, 1937; Hastings & Buchanan, 1942; Saxton & Miller, 1944). Similarly, glucose does not raise the level of respiration when added to liver slices from fasted rats, although such tissue preparations respond well to the addition of sodium pyruvate.

The simplest explanation of these findings would be that the liver glycogen is normally formed by glyconeogenesis from C_3 fragments produced from glucose in the peripheral tissues. Such a mechanism would not involve a liver hexokinase.

SUMMARY

1. Homogenates of several rat tissues have been assayed for hexokinase activity, using the rate of glucose disappearance in the presence of adenosinetriphosphate as a measure of activity.

2. Optimal rates of glucose utilization were obtained when the homogenates were prepared in a potassium phosphate buffer, pH 7·8, containing potassium fluoride, and the incubation medium contained the following components (final concentrations in brackets): glucose (0·0012–0·0024M); potassium adenosinetriphosphate (0·005M); magnesium chloride (0·005M); potassium fluoride (0·05M); potassium phosphate buffer, pH 7·8 (0·04M); potassium chloride (0·042M). The incubation was conducted in air at 30° and varied from 2 to 16 min. duration.

3. Under the above conditions, the amount of glucose utilized was proportional to the volume of homogenate used and in most cases increased linearly with the duration of incubation until at least 80 % of the glucose had disappeared.

4. Results, expressed as $-Q_{glucose}$ (μl. glucose disappearing/mg. dry wt. of tissue/hr.) showed that brain (27·1) was the most active tissue and liver (1·4) the least active.

5. A number of tissues have been considered in the light of the relationship between their hexokinase activities and their physiological functions.

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Blood Clotting: the Function of Electrolytes and of Calcium

By J. E. LOVELOCK and B. M. PORTERFIELD
Medical Research Council, Common Cold Research Unit, Harvard Hospital, Salisbury

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The theory of blood clotting advanced by Morawitz (1904) and Fuld & Spiro (1904) postulated that the presence of free calcium ions was a necessary condition of coagulation. This was based on the evidence of Arthus & Pagés (1890) and Pekelharing (1892) that certain anions, namely, oxalate and citrate, whose calcium salts have low solubility products, inhibit the clotting of blood. Vines (1921), and Stewart & Percival (1928) have commented on the unsatisfactory nature of this evidence, and the recent work of Quick & Stephanini (1948) has cast further doubt on these accepted beliefs. They have suggested that calcium functions in blood clotting, not as a free ion, but in combination with prothrombin as an unionized complex. Also that the activity of citrate is not connected with the low solubility of its calcium salt. If these suggestions are accepted, it is of some interest to consider the alternative theories which can be put forward to explain the anticoagulant activity of oxalate and citrate anions.

(1) Oxalate and citrate ions form complexes with low solubility products with other elements, in particular, iron, cobalt and zinc. If it is assumed that some enzyme incorporating one of these elements is necessary for coagulation, then the anions might lower its activity by removal of an essential element from the enzyme.

(2) The process of coagulation involves the interaction of electrically charged colloidal particles. Ions, particularly multivalent ions, are known to exert an influence on such processes and the activity of oxalate and citrate ions may be due to this effect.

The second theory, namely that ions such as oxalate and citrate might act as anticoagulants by a non-specific salt effect was first proposed by Schmidt (1895). Interest in this possibility was, however, largely dropped when Morawitz (1904) suggested that ionized calcium acted catalytically in the coagulation of blood, and when Sabbatani (1908) explained the action of citrate ions in terms of the low solubility product of its calcium salt.

The only systematic studies of the action of neutral salts on coagulation are those of Glazko & Greenburg (1939), Astrup (1944) and Mommaerts (1945). These authors investigated the anticoagulant activity of anions, including oxalate and citrate ions, on the clotting of purified fibrinogen by thrombin. They showed that in this second phase of blood coagulation, the anticoagulant activity of the anions tested was connected with their valency.

The results reported in this paper were obtained by observing the effect of various anions on the coagulation of whole blood, and by determining the concentration and relative proportions of electrolytes needed to restore the coagulability of salt-free plasma. Many physiological processes are known to be highly sensitive to changes in the concentration and species of ions present in the suspending medium. The results indicate the process of coagulation to be similarly sensitive to disturbances in the electrolyte balance of the blood. In particular they