Studies Involving Enzymic Phosphorylation

5. THE 'ACTIVATION' OF RAT-BRAIN HEXOKINASE BY ERYTHROCYTE LYSATES AND MUSCLE EXTRACTS*

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Weil-Malherbe & Bone (1951*b, c*) reported that the hexokinase activity of an aqueous rat-brain extract could be substantially increased by the addition of a human-erythrocyte lysate or rabbit skeletal muscle extract, neither of which itself possessed any appreciable hexokinase activity. Although the 'activator' was non-dialysable and behaved as a protein, they considered that it could not be identified with 6-phosphofructokinase, myokinase or glyceraldehyde 3-phosphate dehydrogenase.

With glucose as substrate, the product of enzymic phosphorylation, glucose 6-phosphate, is an inhibitor of rat-brain hexokinase (Weil-Malherbe & Bone, 1951*a*); it is clear, therefore, that the activator could exert its effect by removing glucose 6-phosphate from the reaction medium. We have repeated the activation experiments of Weil-Malherbe & Bone (1951*b, c*), using the specific enzymic methods of Slater (1953) for determining spectrophotometrically the amounts of hexose mono- and di-phosphates formed from glucose in this system. Furthermore, we have carried out parallel experiments with 2-deoxy-D-glucose as substrate. These studies have led us to the conclusion that the major source of activation with glucose as substrate is attributable to the 6-phosphofructokinase that is present in the erythrocyte lysates and muscle extracts. In addition, evidence from other types of experiment has implicated two weaker activating effects, one exerted directly on the hexokinase and the other on the 6-phosphofructokinase present in the rat-brain extracts.

EXPERIMENTAL

Materials

Adenosine triphosphate (ATP) was isolated as the barium salt by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948) with the following modification. The whole skinned, eviscerated and decapitated rabbit carcass was used instead of the separated muscle, whereby an increased yield of ATP was obtained. The barium ATP was converted into the potassium salt, a 5% excess of the calculated amount of K_2SO_4 being used, and was stored as a frozen 0.05M solution at -15° .

Reduced diphosphopyridine nucleotide (DPNH) was obtained from C. F. Boehringer and Soehne (Mannheim, Germany).

Hexose monophosphate (HMP), a mixture of glucose 6-phosphate (70%) and fructose 6-phosphate (30%), was prepared by the method of Dubois & Potter (1943). The potassium salt was stored as a 0.07M solution at -15° .

2-Deoxy-D-glucose was a gift from Professor M. Stacey, University of Birmingham. In butanol-acetic acid-water (4:1:5, by vol.) it migrated as a single substance on a paper chromatogram, with R_F 0.35 (cf. Edward & Waldron, 1952).

Serum albumin was a sample of 'Albumen (blood)' obtained from British Drug Houses Ltd.

All other substances were the best available commercial preparations. Glass-distilled water was used throughout.

Preparation of extracts containing enzymes and activators

Rat-brain extract. Adult hooded rats of both sexes were used. Aqueous brain extracts were prepared as described by Weil-Malherbe & Bone (1951*a*), and were used either immediately or after storage in the frozen state at -15° for not longer than 4 hr. From the whole brain of one rat, about 9 ml. of extract were obtained.

Yeast hexokinase was prepared according to Slater (1953), after autolysis by the method of Allfrey & King (1950), and was stored at -15° as a paste in 0.75 saturated $(NH_4)_2SO_4$ containing 1% (w/v) glucose. Its high activity was retained for at least 2 years.

Rabbit-muscle fraction A, containing aldolase, triose phosphate isomerase and L- α -glycerophosphate dehydrogenase, was prepared by the procedure of Racker (1947), with the following modification. In order to destroy any contaminating 6-phosphofructokinase (6-PFK) activity, the product was acidified to pH 6 with 0.1N-HCl and dialysed against several changes of glass-distilled water at 0° for 2-3 days. The fraction that was precipitated between 0.5 and 0.75 saturation with $(NH_4)_2SO_4$ was then separated and stored at -15° . It retained its activity for at least 6 months.

Rabbit-muscle fraction B, containing hexose phosphate isomerase and 6-PFK, was prepared according to Slater (1953). Its activity was stabilized by carrying out the isolation in 0.001M ethylenediaminetetraacetate (EDTA). Preparations of high 6-PFK activity, obtained in this way and dissolved in 0.01M ammonium phosphate buffer, pH 7.6, +0.001M EDTA, remained stable on storage at -15° for 6 months.

Erythrocyte lysate. This was prepared from human blood by the method of Weil-Malherbe & Bone (1951*b*) and remained active for several weeks at -15° .

* Part 4: Long (1955).

Rat-muscle extract was prepared according to Weil-Malherbe & Bone (1951c) and remained active for one week at -15° . Any hexokinase activity could be destroyed completely by heating at 55° for 5 min. and removing the coagulated protein.

Methods

Determination of hexose utilization. Unless otherwise stated, the conditions were similar to those used by Weil-Malherbe & Bone (1951a), except that in place of NaHCO_3 the system was buffered at pH 7.6 with aminotrihydroxymethylmethane (tris) (Gomori, 1946) or potassium phosphate. The reaction mixture normally contained (final concn. in brackets): glucose or 2-deoxyglucose (0.004M), ATP (0.005M), MgCl_2 (0.0067M), KF (0.024M) and tris or phosphate buffer, pH 7.6 (0.025M). Rat-brain extract or a diluted solution of the stock yeast-hexokinase preparation (0.05 ml.), with or without activator (0.05–0.15 ml.), was added; total vol., 0.5 ml. After incubation for 20 min. at 30° , reaction mixtures were deproteinized with the $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ reagents of Somogyi (1945) and glucose was determined in the filtrates by the copper-reduction method of Nelson (1944), according to the procedure described by Long (1951). When 2-deoxyglucose was used as substrate, copper reduction was measured with 'reagent 60' of Shaffer & Somogyi (1933), as recommended by Sols & Crane (1954). When HMP or fructose 1:6-diphosphate (HDP) was to be determined, as described below, reaction mixtures were deproteinized by addition of 0.1 ml. 30% (w/v) trichloroacetic acid (TCA).

Determination of 6-PFK activity. The conditions were identical with those described for hexose utilization, except that potassium HMP (final concn. 0.014M) replaced hexose, and that the ATP concn. was increased to 0.01M. Enzyme activity was stopped by adding 0.1 ml. 30% (w/v) TCA. After diluting to 5 ml. with 5% (w/v) TCA and filtering, a known volume of the neutralized filtrate was analysed for HMP and HDP.

Determination of HMP and HDP. By the use of the appropriate rabbit-muscle fractions, HMP and HDP were converted into dihydroxyacetone phosphate, which oxidizes DPNH in the presence of L- α -glycerophosphate dehydrogenase. The spectrophotometric method of Slater (1953) was used, with two modifications: (i) tris buffer (pH 7.6; final concn., 0.025M) replaced glycylglycine; (ii) it was found necessary to add the ATP and Mg^{2+} to the spectrophotometer cells at the start of the analysis instead of with rabbit-muscle fraction B, as otherwise HMP could

not be satisfactorily determined. It should be noted that HMP, as determined by this method, includes glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate, while part of the measured HDP may consist of triose phosphate.

RESULTS

Enzymic phosphorylation of glucose and 2-deoxyglucose in the absence of activators

Rat-brain extract. Weil-Malherbe & Bone (1951a-c) buffered their reaction mixtures with 0.02M- NaHCO_3 in an atmosphere of $\text{N}_2 + \text{CO}_2$ (95:5). We have found, however, that the nature of the buffer exerts a marked effect on the rate of glucose utilization by a rat-brain extract (Table 1); the rate with bicarbonate buffer was intermediate between those obtained with tris and phosphate.

When a rat-brain extract was incubated with glucose and ATP in a medium buffered with tris (Fig. 1), the following results were obtained. After 4 min., the glucose disappearing was quantitatively converted into HMP; after 8 min., there was little further increase in HMP concentration and some HDP was formed. As incubation continued, the rate of glucose utilization decreased considerably, formation of HDP was almost linear and there was a fall in HMP concentration. This result is consistent with the presence of hexokinase, phosphohexose isomerase and 6-PFK in the brain extract. After 1 hr. about 90% of the glucose disappearing had been converted into HDP. When HMP was used as substrate, there was no disappearance in the absence of ATP, indicating that no side reactions, such as conversion into 6-phosphogluconic acid, were taking place; in the presence of ATP, HMP was converted almost quantitatively into HDP.

Crane & Sols (1954) have shown that 2-deoxyglucose is phosphorylated by 'particulate' ox-brain hexokinase to give 2-deoxyglucose 6-phosphate. This compound is inactive as a substrate for phosphohexose isomerase or 6-PFK; furthermore, unlike glucose 6-phosphate, it does not inhibit rat-

Table 1. *Utilization of glucose and 2-deoxyglucose by rat-brain extract and yeast hexokinase in the presence of different buffers*

Composition of reaction medium (final concn. in brackets): glucose or 2-deoxyglucose (0.004M), ATP (0.005M), MgCl_2 (0.0067M), KF (0.024M) and buffer as indicated. Rat-brain extract (from a centrifuged 1:8 water homogenate) or yeast hexokinase (stock preparation diluted 1:200), 0.05 ml.; total vol., 0.5 ml.; 20 min. incubation at 30° .

Buffer	Final concn. (M)	Rat-brain extract		Yeast hexokinase	
		Glucose used (μ moles)	2-Deoxyglucose used (μ moles)	Glucose used (μ moles)	2-Deoxyglucose used (μ moles)
Tris	0.025	0.25	1.01	0.71	1.08
NaHCO_3^*	0.020	0.36	1.07	0.71	—
Potassium phosphate	0.025	0.81	1.09	0.75	0.94

* Equilibrated with $\text{N}_2 + \text{CO}_2$ (95:5).

Table 2. *Effects of erythrocyte lysate, rat-muscle extract and rabbit-muscle fraction B on the utilization of glucose and 2-deoxyglucose by rat-brain extracts*

Enzyme: rat-brain extract (from a centrifuged 1:8 water homogenate; 0.05 ml.) Activators: human-erythrocyte lysate (washed human erythrocytes, lysed with enough water to restore vol. to that of blood taken; 0.15 ml.); rat-muscle extract (from a centrifuged 1:3 homogenate in 0.03M-NaHCO₃; 0.05 ml.); rabbit-muscle fraction B (stock solution diluted 1:5 and dialysed for 1 hr. against 0.25M tris or potassium phosphate buffer, pH 7.6, at 0°; 0.05 ml.). Enzyme and/or activator incubated for 20 min. at 30° with glucose or 2-deoxyglucose, ATP, MgCl₂, KF and buffer, pH 7.6, at concn. shown in Table 1; total vol. 0.5 ml. In all cases, data for enzyme + activator have been corrected for the slight substrate disappearance due to activator alone.

Expt.	Buffer	Activator	Glucose utilization			2-Deoxyglucose utilization		
			No activator (μmoles)	Activator present (μmoles)	Activation (%)	No activator (μmoles)	Activator present (μmoles)	Activation (%)
1	Tris	Human-erythrocyte lysate	0.22	0.54	145	—	—	—
2	Tris	Rat-muscle extract	0.19	0.74	290	0.78	1.20	54
3	Tris	Rabbit-muscle fraction B	0.25	0.68	172	0.93	1.24	33
4	Phosphate	Rat-muscle extract	0.43	0.68	58	0.86	1.02	19
5	Phosphate	Rabbit-muscle fraction B	0.53	0.74	40	0.99	1.12	13

Fig. 1. Disappearance of glucose and formation of HMP and HDP in rat-brain extract. Composition of reaction medium (final concn. in brackets): glucose (0.004M), ATP (0.005M), MgCl₂ (0.0067M), tris (0.025M) and KF (0.024M). Rat-brain extract (from a centrifuged 1:8 water homogenate), 0.05 ml.; total vol., 0.5 ml.; temp., 30°. ○, Glucose disappearance; ●, HMP formation; □, HDP formation.

brain hexokinase activity. As shown in Table 1, the enzymic phosphorylation of 2-deoxyglucose takes place much more rapidly than that of glucose when the two substrates are compared by using the same rat-brain extract; the difference is most marked when the reaction mixtures are buffered

with tris. The rate of phosphorylation of 2-deoxyglucose was not markedly affected by the nature of the buffer.

Yeast hexokinase. With glucose as substrate, the amount disappearing was quantitatively accounted for as HMP; the enzyme preparation was free from 6-PFK activity. The nature of the buffer had no appreciable effect on the rate of enzymic phosphorylation of either glucose or 2-deoxyglucose; the latter was the more active substrate (Table 1).

Effects of activators on the utilization of glucose and 2-deoxyglucose

Rat-brain extract. Weil-Malherbe & Bone (1951b, c) showed that the rate of glucose utilization by a rat-brain extract could be greatly increased by the addition of a human-erythrocyte lysate or a rabbit-muscle extract, although neither activator had any appreciable hexokinase activity. We have confirmed these observations and find that the rate of utilization of 2-deoxyglucose is also slightly increased in the presence of activators, although the effect produced is only about 20–30% of that obtained with glucose as substrate (Table 2). Rabbit-muscle fraction B also showed similar activating properties. Table 2 shows further that the percentage activation of glucose utilization was always much higher in a tris-buffered medium than in the presence of phosphate; the difference is attributable to the increased rate of glucose disappearance in phosphate-buffered media in the absence of activator (Table 1). It may be concluded from these results that the activators exert their effects to some small extent by their direct action on hexokinase, as shown by the data for 2-deoxyglucose; however, with glucose as substrate the much greater activation observed strongly suggests that they possess a quantitatively more important action.

In the enzymic phosphorylation of glucose, the amounts of HMP and HDP formed have been determined in the presence and absence of activators. In these experiments the reaction medium contained tris, since this buffer favoured large activating effects. The data of Table 3 show clearly that the extra glucose disappearing in the presence of activator is largely accounted for by an increased formation of HDP. Of even greater significance, however, is the observation that the three activators studied all bring about a substantial fall in the level of HMP in the reaction medium. Clearly, this action could cause an

is due to phosphohexose isomerase, however, for Weil-Malherbe & Bone (1951*b, c*) have already shown that both erythrocyte lysates and rabbit-muscle extracts stimulate the utilization of fructose as well as of glucose by rat-brain extract, and we have obtained the same result by using rat-muscle extract as activator. These observations suggest that the activation takes place at a stage beyond phosphohexose isomerase, which would implicate 6-PFK. This possibility is supported by the fact that partially purified 6-PFK (rabbit-muscle fraction *B*) can also cause activation, as shown in Tables 2 and 3.

Table 3. *Effects of activators on glucose utilization and HMP and HDP formation in rat-brain extracts*

Experimental conditions as in Table 2. Reaction mixtures were buffered with 0.025*M* tris and analysed for glucose, HMP and HDP. In all cases the data for enzyme + activator have been corrected by subtraction of the slight effects due to activator alone.

Expt.	Activator		Changes observed (μ moles)			Activation (%)
			Glucose	HMP	HDP	
1	Human-erythrocyte lysate	Enzyme (<i>a</i>)	-0.22	+0.08	+0.18	—
		Enzyme + activator (<i>b</i>)	-0.54	+0.01	+0.46	—
		(<i>b</i>) - (<i>a</i>)	-0.32	-0.07	+0.28	145
2	Rat-muscle extract	Enzyme (<i>a</i>)	-0.42	+0.12	+0.19	—
		Enzyme + activator (<i>b</i>)	-0.90	+0.06	+0.64	—
		(<i>b</i>) - (<i>a</i>)	-0.48	-0.06	+0.45	114
3	Rabbit-muscle fraction <i>B</i>	Enzyme (<i>a</i>)	-0.22	+0.08	+0.18	—
		Enzyme + activator (<i>b</i>)	-1.06	+0.01	+0.72	—
		(<i>b</i>) - (<i>a</i>)	-0.84	-0.07	+0.54	282

Table 4. *Effect of heated and unheated rat-muscle extract and rabbit-muscle fraction B on the utilization of glucose and 2-deoxyglucose*

In Expts. 1-4, rat-brain extract was used; in Expt. 5, the enzyme was yeast hexokinase. Rat-muscle extract or rabbit-muscle fraction *B* was heated for 5 min. at 80°, and the coagulum dispersed by homogenization. Other conditions as in Table 2. The percentage activation is given in brackets.

Expt.	Activator	Buffer	Glucose utilization (μ moles)			2-Deoxyglucose utilization (μ moles)		
			No activator	Unheated activator	Heated activator	No activator	Unheated activator	Heated activator
1	Rat-muscle extract	Tris	0.14	1.01 (620)	0.26 (86)	0.67	1.25 (87)	0.81 (21)
2	Rat-muscle extract	Phosphate	0.47	0.76 (62)	0.57 (21)	0.95	1.28 (35)	1.07 (13)
3	Rabbit-muscle fraction <i>B</i>	Tris	0.25	0.68 (172)	0.29 (16)	0.93	1.24 (33)	0.94 (1)
4	Rabbit-muscle fraction <i>B</i>	Phosphate	0.57	0.91 (60)	0.62 (9)	1.04	1.48 (42)	1.19 (14)
5	Rat-muscle extract	Tris	0.79	1.14 (44)	0.80 (1)	0.95	1.21 (27)	1.00 (5)

indirect stimulation of glucose utilization, since glucose 6-phosphate is an inhibitor of rat-brain hexokinase (Weil-Malherbe & Bone, 1951*a*; Crane & Sols, 1953). If the phosphohexose isomerase or 6-PFK activities of the unsupplemented rat-brain extract limits the rate of removal of glucose 6-phosphate, then the presence of either or both of these enzymes in the activator could produce the observed result. It seems unlikely that activation

Yeast hexokinase. Weil-Malherbe & Bone (1951*b, c*) found yeast hexokinase, with glucose as substrate, to be 32% activated by both human-erythrocyte lysates and rabbit-muscle extracts. Since this enzyme is not inhibited by HMP (Berger, Slein, Colowick & Cori, 1946), the effect clearly cannot be related to the presence of 6-PFK in the activators, and is probably of a similar nature to the slight activation of 2-deoxyglucose utilization

found with rat-brain extracts (Table 2), which was regarded as a direct effect on the hexokinase enzyme. We have confirmed the findings of Weil-Malherbe & Bone, using rat-muscle extract as activator, with both glucose and 2-deoxyglucose as substrate (see Table 4, Expt. 5).

Effect of heat treatment on the activators

Weil-Malherbe & Bone (1951*b, c*) showed that heating activator solutions for 5 min. at 80° destroyed their 6-PFK activity and diminished but did not completely eliminate their effects on glucose utilization by rat-brain extracts. This observation was taken to indicate that activation was not due to 6-PFK activity. We have repeated these experiments, but find that the heat treatment causes a greater fall in activation than that reported by Weil-Malherbe & Bone. Table 4 shows the results obtained with both glucose and 2-deoxyglucose as substrates. With glucose, the effect of the activator was much reduced by heat treatment, being especially marked when the system was buffered with tris. With 2-deoxyglucose, the heated activators had practically no effect on substrate utilization.

With yeast hexokinase, heated rat-muscle extract had practically no activating effect on the utilization of glucose or 2-deoxyglucose (Table 4).

Activation by heat-denatured serum albumin and EDTA

By the use of rat-brain extract with glucose as substrate, it has been found that activating effects are produced by serum albumin and EDTA (Table 5). It will be observed that the percentage activations caused by EDTA and heat-denatured

albumin are of the same order of magnitude as those caused by heat-treated rat-muscle extract. By contrast, it was found that when 2-deoxyglucose was the substrate, EDTA and heat-denatured albumin had little or no activating effect.

6-PFK activities of rat-brain extract and activators

6-PFK activities were measured by incubating rat-brain extract or activator with 0.014*M* HMP and 0.01*M* ATP, as described in the Methods section, and determining loss of HMP and formation of HDP. This concentration of substrate is more than sufficient to saturate the enzyme (Weil-Malherbe & Bone, 1951*a*), but raising the ATP concentration above 0.01*M* did not increase the rate of utilization of HMP. These conditions of assay were not necessarily optimal, but it was clearly desirable to keep them as similar as possible to those used in the activation experiments. Disappearance of HMP was in all cases quantitatively accounted for as HDP, and no dephosphorylation was observed. Relative 6-PFK activities are given in Table 6. Rat-muscle extract and the 1:5-diluted rabbit-muscle fraction *B* caused a utilization of 3–3.5 μ moles HMP/0.05 ml./30 min. Human-erythrocyte lysate and rat-brain extract possessed lower activity. It is noteworthy that the activation of rat-brain hexokinase by erythrocyte lysate is usually rather less than that produced by either rabbit-muscle fraction *B* or rat-muscle extract, which is consistent with the suggestion that the main activator is 6-PFK.

In an experiment with rat-brain extract, activated by either erythrocyte lysate, rat-muscle extract of low 6-PFK activity (caused by filtration

Table 5. *Effects of heat-denatured serum albumin, EDTA and acid-treated activators on the utilization of glucose by rat-brain extract*

Serum albumin solution, denatured by heating for 5 min. at 80°; final concn. 0.5%. EDTA, final concn., 0.001*M*. Rat-muscle extract or rabbit-muscle fraction *B* was acidified to pH 6 and left at room temp. for 1 hr., and the pH was then readjusted to 7.6. In Expts. 3 and 4, the figures in brackets denote the 6-PFK activities of the muscle extracts (μ moles HMP utilized/20 min.).

Expt.	Buffer	Activator	Glucose utilization (μ moles)		
			No addition	Activator present	Activation (%)
1	Phosphate	Rat-muscle extract	0.47	0.91	94
		Heated rat-muscle extract	0.47	0.57	23
		Albumin	0.47	0.56	19
		EDTA	0.47	0.67	43
2	Tris	Rat-muscle extract	0.27	0.94	248
		Heated rat-muscle extract	0.27	0.49	81
		Albumin	0.27	0.41	52
		EDTA	0.27	0.43	59
3	Tris	Rat-muscle extract	0.32	1.00 (2.53)	212
		Acid-treated rat-muscle extract	0.32	0.68 (1.17)	115
4	Phosphate	Rabbit-muscle fraction <i>B</i>	0.63	1.69 (2.89)	170
		Acid-treated rabbit-muscle fraction <i>B</i>	0.63	0.77 (0.05)	22

through a thick pad of kieselguhr), fraction *B* of high 6-PFK activity, and various combinations of these, the degree of activation roughly paralleled the total 6-PFK activity of the added activators (Table 7).

Effects of nature of buffer, EDTA, heated and acidified activators and serum albumin on the 6-PFK activity of rat-brain extracts

The heated activators, heat-denatured serum albumin and EDTA all increase the rate of glucose utilization by rat-brain extracts (Table 5). These effects could be exerted on the hexokinase and/or

6-PFK activities of the brain extract. Table 8 shows that all the substances mentioned above increase the measured 6-PFK activity of rat-brain extracts. The 6-PFK activity of rat-brain extracts is also greater in the presence of phosphate buffer than in tris buffer. Since 6-PFK activity is sensitive to mild acid treatment at room temperature (Taylor, 1947; Utter, 1947), the effect of acidification on the activators was of particular interest. As will be seen from Expts. 3 and 4 of Table 5, the diminution of the activating power of rat-muscle extract and rabbit-muscle fraction *B* paralleled the reduction in 6-PFK activity.

Table 6. 6-PFK activities of rat-brain extract and activators

Rat-brain extract or activator was incubated with 0.014M HMP, 0.01M ATP, 0.0067M-MgCl₂, 0.024M-KF and 0.025M tris buffer, pH 7.6, for 20 min. at 30°; total vol. 0.5 ml. Reaction mixtures deproteinized with TCA and filtrates analysed for HMP and HDP.

Source of 6-PFK	Vol. used (ml.)	No. of measurements	Average HMP utilized (μmoles)	Average HDP formed (μmoles)
Rabbit-muscle fraction <i>B</i> *	0.05	8	3.42	3.41
Rat-muscle extract	0.05	3	3.28	3.27
Human-erythrocyte lysate	0.15	5	1.40	1.33
Rat-brain extract	0.05	7	1.14	1.08

* A 1:5 dilution of stock preparation, dialysed against 0.25M tris buffer, pH 7.6, for 1 hr. at 0°.

Table 7. Glucose utilization by rat-brain extract in the presence of activators of different 6-PFK activities

Rat-brain extract, erythrocyte lysate and rabbit-muscle fraction *B*, as in Table 2; rat-muscle extract was filtered through a thick pad of kieselguhr in order to diminish its 6-PFK activity. Tris buffer used; other conditions as in Table 2. Glucose-utilization values are corrected for the slight hexokinase activities of the activators.

Activator	6-PFK activity of activator (μmoles HMP utilized/20 min.)	Glucose utilization (μmole/20 min.)	Activation (%)
None	—*	0.31	—
Rat-muscle extract (<i>a</i>)	0.63	0.41	35
Erythrocyte lysate (<i>b</i>)	1.63	0.49	60
(<i>a</i>) + (<i>b</i>)	2.26	0.62	103
Rabbit-muscle fraction <i>B</i> (<i>c</i>)	2.46	0.76	149
(<i>a</i>) + (<i>c</i>)	3.09	0.81	164
(<i>a</i>) + (<i>b</i>) + (<i>c</i>)	4.72	0.85	178

* 6-PFK activity of rat-brain extract alone, 1.90 μmoles HMP utilized/20 min.

Table 8. Effects of buffer, heated rat-muscle extract, serum albumin and EDTA on the 6-PFK activity of rat-brain extracts

Experimental conditions as in Table 6. 20 min. incubation at 30°.

Expt.	Buffer	Activator	HMP utilized (μmoles)	HDP formed (μmoles)
1	Tris	None	1.95	1.44
	Phosphate	None	2.56	2.68
2*	Phosphate	None	0.87	0.93
		Heat-denatured albumin	1.26	1.30
		Heat-treated rat-muscle extract	1.37	1.48
		EDTA	1.64	1.78

* A diluted rat-brain extract was used in this experiment.

Stern (1954) has stated that the rate of utilization of glucose by a rat-brain extract, both in the presence and absence of activator, is lowered by the addition of 0.003M-CaCl₂. We have found that under these conditions the 6-PFK activity of the rat-brain extract is diminished, and this could partly explain the effects reported. In addition, however, Ca²⁺ ions are known to form a complex with ATP (DiStefano & Neuman, 1953), so that the reduced activity is probably also partly attributable to this cause.

Inhibition of 2-deoxyglucose utilization in acid-treated rat-brain extract by HMP and stimulation by rat-muscle extract

If activators increase the rate of glucose utilization in rat-brain extracts largely by removal of inhibitory HMP by virtue of their 6-PFK activity, then it should be possible to demonstrate a similar effect with 2-deoxyglucose as substrate in the presence of added HMP. Under these conditions, there would be no formation of HMP during the experiment, in contrast with what would occur with glucose as substrate. In order to prevent the

phosphorylating glucose (Fig. 1 and Table 3), inhibited 2-deoxyglucose utilization by about 47%. When rat-muscle extract was added, the inhibition almost completely disappeared and the apparent activation was 173%, compared with 52% for the uninhibited system.

DISCUSSION

The observations first made by Weil-Malherbe & Bone (1951*b, c*) that lysates of human erythrocyte and rabbit-muscle extracts were able to increase the rate of glucose utilization by a suitably supplemented rat-brain extract seem now to be satisfactorily explained in terms of the enzymic pathway: glucose → glucose 6-phosphate → fructose 6-phosphate → fructose 1:6-diphosphate.

The elucidation of the mechanisms involved has been due mainly to the use of 2-deoxyglucose as an alternative substrate for rat-brain hexokinase, since in this way the hexokinase reaction becomes isolated from subsequent stages of the glucolytic pathway. The employment of the spectrophotometric method of Slater (1953) for the specific determination of hexose mono- and di-phosphates and the observations made in the presence of different buffers have also materially helped in investigating this problem.

It seems quite clear that the activators do have a slight effect on the true hexokinase activity of rat-brain extracts. This is shown by the fact that an activation was obtained with 2-deoxyglucose as substrate (Table 2). Activations of the same order of magnitude (20–50%) were also observed with yeast hexokinase, both glucose and 2-deoxyglucose being used as substrates (Table 4).

These activations are much smaller than those observed by Weil-Malherbe & Bone and by ourselves with rat-brain extract, using glucose as substrate; these usually amounted to about 150–300% (Table 2), but were sometimes considerably higher. Such high values are traceable to the potent 6-phosphofructokinase contained in the activators; this enzyme acts by causing the rapid removal of fructose 6-phosphate, which consequently reduces the concentration of glucose 6-phosphate, a potent inhibitor of brain hexokinase. The main evidence in support of this explanation is as follows: (1) the activators lower the concentration of hexose monophosphate; (2) they convert most of the extra glucose utilized into fructose 1:6-diphosphate; (3) they have high 6-PFK activities; (4) their effect on the rate of glucose utilization is roughly proportional to their 6-PFK activity; (5) they remove the inhibition of 2-deoxyglucose utilization caused by added HMP; and (6) any treatment which lowers their 6-PFK activity also lowers their activating effects.

Table 9. *Effects of HMP and rat-muscle extract, singly and together, on the rate of 2-deoxyglucose utilization by a rat-brain extract free from 6-PFK activity*

Aqueous rat-brain extract, adjusted to pH 5.5 with N-HCl (glass electrode), incubated 15 min. at 37° and pH readjusted to 7.6 with 3.5N-KOH. Other experimental conditions as in Table 2. HMP, 0.12 μmole where indicated.

Additions	2-Deoxyglucose utilized (μmoles/ 20 min.)	Activation (%)
None	0.83	—
Rat-muscle extract	1.26	52
HMP	0.44	—
Rat-muscle extract + HMP	1.20	173

utilization of the added HMP during the course of the experiment, it was necessary to use an acid-treated rat-brain extract of negligible 6-PFK activity. This was obtained by pre-incubation of the rat-brain extract at pH 5.5 and 37° for 15 min. (Racker & Krimsky, 1948), followed by readjustment of the pH to 7.6; in the experiment shown in Table 9, 0.12 μmole of HMP was added initially and 0.11 μmole was still present at the end of 20 min. incubation with the pre-treated rat-brain extract, showing that the 6-PFK activity had been virtually destroyed by the acid treatment. It will be seen that 0.12 μmole HMP, which is of the same order as the amount normally present in rat-brain extracts

Weil-Malherbe & Bone (1951*b*, *c*) had earlier concluded that 6-PFK was not responsible for the observed effects, since heat treatment of the activators destroyed the 6-PFK activity without eliminating completely their effects on glucose utilization. This is now explained by the fact that the heat-denatured activators stimulate the 6-PFK activity of the rat-brain extracts (Table 8) and so produce a qualitatively similar effect, though of lesser magnitude; we have observed these effects also with denatured serum albumin and with EDTA. Thus there appear to be three mechanisms of activation: (1) direct stimulation of hexokinase activity; (2) addition of 6-PFK contained in the activator solutions; (3) stimulation of the 6-PFK activity of the rat-brain extract. In general, of the total stimulation of glucose utilization due to the addition of activator, 20% was caused by (1), 60% by (2) and 20% by (3).

It will be noted (Table 4) that the rate of utilization of glucose by a rat-brain extract supplemented by addition of an activator is always less than the rate with 2-deoxyglucose as substrate under otherwise identical experimental conditions. Crane & Sols (1954), however, have stated that the two substrates have equal maximal rates of utilization with particulate ox-brain hexokinase, when the substrate concentrations are sufficient to saturate the enzyme, as in the present work. The explanation of this discrepancy may be traceable to the inhibition of rat-brain hexokinase by ADP (Weil-Malherbe & Bone, 1951*a*; Sols & Crane, 1954), the concentration of which would be greater, with glucose as the substrate than with 2-deoxyglucose. On the other hand, it is possible that the 6-PFK activity of the activators was insufficient to ensure maximal glucose utilization.

The stimulating effect of phosphate ions on the utilization of glucose by rat-brain extract (Table 1) seems undoubtedly due to the increased 6-PFK activity in this buffer (Table 8); such an explanation also accounts for the fact that the effects of the activators, both heated and unheated, were always uniformly less when this buffer was employed in place of tris (Table 4). Phosphate has no appreciable effect on hexokinase itself, as shown with rat brain extract with 2-deoxyglucose, and with yeast hexokinase with either glucose or 2-deoxyglucose.

SUMMARY

1. The 'activation' of glucose utilization in rat-brain extracts by erythrocyte lysates and rat-muscle extracts has been studied.

2. A slight direct activation of rat-brain hexokinase was observed when 2-deoxyglucose was

used as substrate; similar effects were found with the yeast enzyme, with both glucose and 2-deoxyglucose as substrates.

3. Quantitatively the most important effect is due to the increased 6-phosphofructokinase activity of the enzyme system, brought about by addition of activator. This acts by removing inhibitory HMP from the reaction medium.

4. A third effect is exhibited by the stimulation of the 6-phosphofructokinase activity of the rat-brain extract by heat-denatured activators, none of which possessed any detectable 6-PFK activity. Similar effects were produced by heat-denatured albumin, EDTA and phosphate ions.

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