Biochemical Pharmacology, 1962, Vol. 11, pp. 803–807. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF VARIOUS AGENTS *IN VIVO* ON PHOSPHORYLASE ACTIVITY OF RAT BRAIN

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(*Received* 24 *October* 1961; *accepted* 10 *May* 1962)

Abstract—The phosphorylase enzyme, which catalyzes the conversion of glycogen to glucose-1-phosphate, appears to have its own activator(s) and possibly inhibitor(s) according to the source of the enzyme. Several drugs were tested in a search for substances that increase or decrease the level of activity of this enzyme in the brain. Fed or fasted rats were injected subcutaneously with the drugs and, 45–75 minutes later, the brains were removed and tested for enzyme activity. The values of the control group indicated that the phosphorylase of the brain is predominantly in the active form *a*. The activity of the enzyme was unaffected by starving. Of the substances tested, only insulin was able to enhance activity, and this increase was achieved only with drastic treatment—*i.e.* a near-convulsive dose plus 24-hr fasting. Both D-amphetamine and L-epinephrine decreased the enzyme activity significantly. Other substances tested (L-DOPA, ACTH, cortisone, hydrocortisone, and chlorpromazine) had no effect.

PHOSPHORYLASE, the enzyme involved in the conversion of glycogen to glucose-1-P* is reportedly implicated in the contraction of the heart,[1–3] adrenocortical activity,[4, 5] glycogenolysis in the liver,[6, 7] and increased metabolism of the liver fluke, *Fasciola hepatica*.[8] In each instance some substance activated the phosphorylase, but the specificity of the activating substances seems to vary with the source of the enzyme. Thus, adrenocortical phosphorylase is activated by ACTH,[4, 5] and liver phosphorylase by glucagon and epinephrine[6, 7] but not by ACTH. Heart phosphorylase is activated by sympathomimetic amines such as isoproterenol, norepinephrine, epinephrine, and epinine, in decreasing order of potency.[2] In *F. hepatica* serotonin is the activating substance.[8]

From the foregoing evidence it was assumed that brain phosphorylase could be activated or inactivated by specific substances. It was the purpose of this investigation to determine which substances behave as activators or inhibitors on phosphorylase activity of the rat brain *in vivo*.

METHODS AND MATERIALS

The following drugs were used in this investigation: insulin (Illetin), D-amphetamine HCl, L-epinephrine bitartrate, ACTH, cortisone (cortisone acetate aqueous suspension), hydrocortisone (Solu-Cortef), and chlorpromazine HCl. The liquid preparations were either undiluted or diluted with saline. Solid drugs were dissolved in saline.

* Abbreviations: glucose-1-P (dipotassium glucose-1-phosphate); ACTH (adrenocorticotropic hormone); EDTA (disodium ethylenediamine tetraacetate); AMP (adenosine-5′-phosphate); L-DOPA, (L-dihydroxyphenylalanine).

Other chemicals and solutions were NaF (0·1 M)-EDTA (0·005 M); β-glycerophosphate (0·035 M, pH 6); glucose-1-P (0·064 M, pH 6); glucose-1-P (0·064 M, pH 6) containing AMP (0·004 M); and glycogen (4%).

Wistar strain albino rats of etither sex, weighing 100 to 200 g were used in a well fed or fasted state. The fasted rats were originally well fed animals which had been deprived of food for 24 hr unless otherwise indicated. All drugs were administered subcutaneously and animals were usually sacrificed 45 to 75 min later. The brain was removed as quickly as possible (about 2 min) and immediately placed in a freezer for at least 30 min.

For the phosphorylase determination the brain was cut symmetrically in half and either section was used. It was weighed and homogenized in a cold Tenbroeck tissue grinder with 5 volumes of cold distilled water. The homogenate was centrifuged in the cold at 7000 × *g* for 20 min, at which time the supernatant was decanted through glass wool into a cold test tube. A definite volume of the supernatant was mixed immediately with an equal volume of cold NaF-EDTA solution. This mixture was used in the assay of the phosphorylase enzyme. The procedure was a modification of the method of Cori *et al.*[9] as follows: to a test tube containing 0·2 ml of glycogen and 0·3 ml of β-glycerophosphate, 0·1 ml of the enzyme solution was added and incubated for 20 min at 30 °C. Then 0·2 ml of glucose-1-P with or without AMP was added and incubated for 15 min at 30 °C. The liberated inorganic phosphate was determined by the method of Fiske and Subbarow as described by Lindberg and Ernster[10] using a 0·2-ml aliquot at zero time and at 15 min. The activity was expressed in phosphorylase units[11] per ml of the enzyme solution, whose concentration is equal to dilution of the tissue in 10 volumes of liquid. The phosphorylase units were calculated according to the following formula: Phosphorylase units $= 100 \times 1/t \times \log Xe/(Xe - X)$, where $t = 15$ min and $Xe =$ per cent conversion of glucose-1-P at equilibrium. The value used for Xe was 85. $X =$ per cent of the initial glucose-1-P which had reacted after 15 min of incubation. This value should be preferably below 15 per cent. The ratio, units of phosphorylase without AMP/units of phosphorylase with AMP × 100 is a measure of the relative amount of the active form *a* to the total phosphorylase. The value of 65 per cent would indicate that the enzyme is completely in the active form *a*.[11]

RESULTS

The results of the experiments are summarized in Table 1. The foregoing breakdown indicates that the phosphorylase enzyme of the whole brain of rat is predominantly in the active form *a* as indicated by the per cent ratio of 58 for the control group. There was no apparent difference between the fed and fasted rats given saline so that they were not separated in the control group. The total activity—*i.e.* the one determined in the presence of AMP—is rather constant throughout, and no agents tested affected the level significantly. Only one substance, insulin, was capable of increasing the active form and consequently the ratio. Even this was achieved only with drastic measures. The rats were starved 24 hr and near-convulsive doses had to be administered. Fed animals or those starved for 17 hr or less did not show increase in enzyme activity. Both D-amphetamine and L-epinephrine significantly decreased the level of the active form of the enzyme, the former being much more effective. The ACTH, which is known to stimulate the phosphorylase enzyme of the adrenocortex,[4, 5] had no effect whatsoever, either in fed or starved rats. Hydrocortisone was also ineffective. Other

TABLE 1. EFFECTS OF VARIOUS AGENTS *IN VIVO* ON PHOSPHORYLASE ACTIVITY OF RAT BRAIN*

For the assay the brain was prepared as described in Methods and Materials. The sample contained 0·05 M NaF and 0·0025 M EDTA. The assay system consisted of 0·1 ml sample; 0·3 ml 0·035 M β-glycerophosphate, pH 6·0; 0·2 ml 4% glycogen; and 0·2 ml 0·064 M glucose-1-P with or without 0·004 M AMP. The samples were incubated for 15 min at 30 °C. Results were compared with the group treated with saline.

Substances administered	N	Dosage range per kg	F or S	− AMP(A) Phosphorylase units	*t*	*P*	+ AMP(B) Phosphorylase units	*t*	*P*	Ratio A/B × 100	*t*	*P*
Saline	31	5 ml	Mixed	127 ± 2·4			218 ± 3·0			58·21 ± 0·73		
Insulin	7	16–32 units	S	142 ± 5·3	2·59	< 0·02	213 ± 6·2	0·80	< 0·5	66·67 ± 2·05	4·70	< 0·001
Insulin	6	16–32 units	F or Partly S	135 ± 4·0	1·34	< 0·2	223 ± 4·3	0·70	< 0·5	60·45 ± 1·06	1·31	< 0·2
D-Amphetamine	8	5–10 mg	S	103 ± 5·3	4·40	< 0·001	210 ± 3·8	1·42	< 0·2	49·10 ± 2·10	5·15	< 0·001
L-Epinephrine	6	2·5–5 mg	S	111 ± 4·4	2·76	< 0·01	220 ± 2·6	0·21	⩾ 0·5	50·50 ± 1·91	4·17	< 0·001
ACTH	6	10–20 units	F	123 ± 4·9	0·70	< 0·5	221 ± 5·7	0·34	⩾ 0·5	55·70 ± 0·97	1·46	< 0·2
ACTH	6	10–20 units	S	124 ± 4·8	0·44	⩾ 0·5	214 ± 6·3	0·58	> 0·5	58·10 ± 1·88	0·06	⩾ 0·5
Hydrocortisone	5	20 mg	S	120 ± 3·7	1·21	< 0·3	208 ± 5·0	1·35	< 0·2	57·40 ± 1·05	0·43	⩾ 0·5

* N = number of animals; F = fed; S = starved 24 hr; Partly S = starved less than 24 hr (usually overnight); − AMP = without added AMP (phosphorylase activity in phosphorylase units per unit volume); + AMP = with added AMP (units as above); t = Student's t test; P = probability.

substances, although not included in the table, such as cortisone, chlorpromazine, and L-DOPA showed no indication of activity. The effect of chlorpromazine is in accord with a recent investigation.[12]

DISCUSSION

The results described in this investigation indicate that the activity of the brain phosphorylase can be either increased or decreased by certain substances. It should be borne in mind, however, that these effects could be secondary. Insulin increased the activity of the enzyme under certain conditions, whereas D-amphetamine and L-epinephrine decreased the active form of the enzyme. Brain phosphorylase is, therefore, similar to phosphorylases from other sources, because its activity can be accelerated by an activator substance. The peculiarity of this phenomenon is illustrated by the fact the activator substance for the enzyme from one source may not necessarily be active for the enzyme from another source. Thus, phosphorylase from different sources may require different systems for its activation.

This explanation may also apply to the agents that depress activity, but no comparison can be attempted at this time since insufficient information is available in the literature on this subject. More extensive data on the inhibitory as well as the activating substances on phosphorylase from different sources might give a better view of the specificities and mechanisms involved.

Some comments are in order regarding the methods used in this investigation. The original method chosen in this study for the demonstration of phosphorylase was that of Cori and Illingworth as modified by Hess and Haugaard.[1] Brain tissue assayed for phosphorylase by this method showed a per cent ratio of *a* to total enzyme of 60 to 70. By the interpretation of Cori and Green[11] this would indicate that brain phosphorylase is predominantly in the active *a* form. If the interpretation is correct then it can be assumed that brain phosphorylase exists in the maximally active state or that the results are an artifact attributable to the method of determination. Should the former be true then no further increase in activity could be effected by the addition of any substance, whereas if the latter is true then activator substance or substances might exist without being detected.

In an attempt to detect agents that would further increase phosphorylase activity it was decided to modify the procedure. When brain tissue was homogenized in ice-cold water and the supernatant mixed with the solution of sodium fluoride and EDTA, the per cent ratios were about 10 per cent lower than before. When identical halves of a brain were assayed by these two methods, the aqueous method yielded consistently higher values in the presence of AMP and about equal values in its absence, resulting in lower ratios for this method. Whether this resulted from more efficient extraction of the enzyme by water accompanied by partial conversion of *a* to *b* or to faster inactivation of *b* than *a* in the NaF-EDTA extraction method was not known, although both possibilities existed. The water extraction method was chosen only because it seemed to offer greater possibility of detecting increased phosphorylase activity.

The still relatively high ratio obtained by the aqueous method could hardly have been due to endogenous AMP. To minimize its effect, the homogenate was diluted 1 to 80 in the incubation mixture for the determination of the enzyme activity. At this dilution, the concentration of all substances that show absorption at 260 mμ, calculated as AMP, is in the neighborhood of 5×10^{-5} M which is a dissociation constant for the

complex of phosphorylase *b* and AMP.[13] Since such substances cannot all be AMP, their effect, if any, would be small.

The inorganic phosphate liberated in the incubation tube was due to conversion of glucose-1-P to glycogen. This was evident from the fact that the release of inorganic phosphate was negligible in the absence of glycogen.

The effect of the time lapse between removal and freezing of the brain on the phosphorylase activity was also considered. True enzyme values are difficult to determine, since freezing *per se* is reported to affect the enzyme level,[7] no matter how quickly the tissue is frozen. In the beginning of this investigation the brain tissues were homogenized immediately after removal in cold homogenizing fluid but, because of the inconvenience in so processing a number of animals at one time, the freezing procedure was adopted. There were no apparent differences in the enzyme activity between the two methods. Since we were interested in detecting substances which either enhance or depress the enzyme activity, the uniformity of procedure was more important than obtaining the true absolute values. No strong claim is made for the accuracy of the figures obtained with respect to the true *in vivo* values.

One might note that there is an unusually large number of samples in the control group (saline). This was due to intermittent testing with saline in order to insure that the different shipments of drugs and other variables would have minimal effect on the results obtained.

Acknowledgement—The authors wish to thank Irving J. Blumenthal, M.D., Associate Chief of Staff, for providing the facilities for this research and for giving invaluable assistance in editing the manuscript.

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