Biochemical Pharmacology, 1961, Vol. 6, pp. 189–194. Pergamon Press Ltd., Printed in Great Britain.

PHOSPHORYLASE ACTIVITY IN HEART AND BRAIN AFTER RESERPINE, IPRONIAZID AND OTHER DRUGS AFFECTING THE CENTRAL NERVOUS SYSTEM*

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(*Received* 26 *July* 1960; *in revised form* 26 *October* 1960)

Abstract—Phosphorylase activity in heart and brain was determined after treatment of intact rats with several agents affecting the central nervous system. Reserpine decreases the percentage of phosphorylase *a* in the heart. There is suggestive but non-conclusive evidence that, under certain conditions, reserpine may also lower the phosphorylase *a* content of the brain. Iproniazid pretreatment does not prevent, and, in fact, may enhance the diminution of phosphorylase *a* in the heart and brain of reserpinized animals. Iproniazid alone produces small but non-significant increases of phosphorylase *a* in heart and brain. Serotonin, chlorpromazine and pentobarbital have no effect on brain or heart phosphorylase *a*.

ALTHOUGH many studies have been made of phosphorylase in skeletal muscle,[1–3] relatively little is known concerning this enzyme in cardiac tissue. Phosphorylase is the enzyme which catalyzes the reaction: glycogen + inorganic phosphate ⇋ glucose-1-phosphate.[4] It has been obtained from skeletal muscle in two forms; as a euglobulin form *a* (active) which has 60–70 per cent of its full activity without addition of adenylic acid and as form *b* (inactive) which requires the presence of adenylic acid as co-factor to elicit activity.[5] In skeletal muscle, the predominant phosphorylase is the *b* form.[6]

Following a report on the purification of phosphorylase from dog heart, in which the enzyme exists in both an active and an inactive form,[7] and the observation on the high phosphorylase *a* content of heart muscle,[2] more recent studies have appeared which indicate that functional activity, drugs and hormones may affect phosphorylase activity in the heart. Among the several observations, there is complete agreement that epinephrine and other inotropic catecholamines increase phosphorylase *a* content in cardiac tissue[8–12] possibly through their action in promoting the formation of an essential cyclic nucleotide which is necessary for phosphorylase activation.[13]

Inasmuch as phosphorylase *a* levels may be dependent on intrinsic catecholamines, it would be interesting to study the effects of reserpine on heart phosphorylase *a*, since that agent is capable of nearly depleting cardiac catecholamines.[14] A recent report indicates that, concomitant with a negative chronotropic action, there is no change in phosphorylase *a* in the isolated guinea pig atrium treated with reserpine.[15]

Not only should heart be subject to such studies, but also brain, wherein reserpine causes a release of catecholamines.[16] The investigations on brain would also provide quantitative control data on phosphorylase *a* which, to our knowledge, have not been reported for that tissue.

* Supported by Research Grant H-3724 from the National Heart Institute of the National Institutes of Health.

For comparison with reserpine, experiments were also performed with several other agents affecting the central nervous system.

METHOD

After appropriate intraperitoneal treatment (see tables), male Wistar strain rats weighing approximately 300 g were decapitated. The head was placed immediately in liquid nitrogen, as was the rapidly excised whole heart. To remove brain cortex, the frozen head was sagittaly split and cortical material chipped out with a carpenter's chisel. The tissue was weighed in the frozen state, then placed in ice-cold medium containing 0·001 M Versene and 0·02 M NaF, and immediately homogenized. The supernatant fraction from the homogenate was assayed.

The phosphorylase assay

The method of Cori and Illingworth[17] was employed, as in previous studies.[9, 12] In brief, the determination of phosphorylase *a* and *b* in mixtures is based on two parallel activity measurements, one without and one with the addition of adenylic acid. In the former case, phosphorylase *a* shows on the average 65 per cent of its full activity, while phosphorylase *b* is inactive; in the latter case, phosphorylase *a* and *b* show full activity.

Phosphorylase activity is measured in the direction of glycogen synthesis by determination of the amount of inorganic phosphate formed from glucose-1-phosphate. Results are expressed as μmoles of inorganic phosphate formed and the ratio:

$$\frac{\text{inorganic P formed without adenylic acid}}{\text{inorganic P formed with adenylic acid}} \times 100$$

is designated in this paper as per cent of active phosphorylase *a*.

Effect of freezing tissue samples. It has been reported that freezing in a CO_2–ethanol slush may cause a breakdown of ATP and consequent carry over of significant amounts of adenylic acid into the assay, giving rise to artifactually high phosphorylase *a* values.[10] In a previous study carried out in this laboratory,[12] it was found that the phosphorylase *a* activity of rat atria which had not been frozen was in the same range as that reported for rat ventricles which had been frozen with a CO_2–ethanol slush. Furthermore, in the present study a comparison was made between the phosphorylase *a* content of rat ventricles (5) which had not been frozen, with samples which had been frozen in liquid nitrogen. The two types of treatment did not result in phosphorylase *a* values which were appreciably different. In the case of brain, there was some delay in the removal of non-frozen tissue, and a comparison of frozen tissue with fresh tissue was not made. Brain and heart tissue samples which had been diluted 1 : 160 were compared with samples which were diluted 1 : 40, as in our standard procedure; there was no marked difference in their respective phosphorylase *a* activity. This would suggest that the amount of endogenous adenylic acid present was not great enough to influence the results.

Centrifugation. Heart and brain homogenates were centrifuged for 15 min at 900 *g* at 0 °C. Preliminary studies indicated that this centrifugation did not result in an appreciable loss of phosphorylase with the discarded precipitate.

Phosphatase controls. Four determinations each, on brain and heart, in which glycogen primer was omitted from the assay system, indicated that phosphatase activity made negligible contributions to inorganic phosphate release. No corrections were

applied since these were sufficiently minor as not to change the nature or the interpretation of the data. Under certain conditions, otherwise negligible but constant corrections may tend to lower phosphorylase *a* values; thus, it is considered advisable to check this point in each investigation.

TABLE 1. PHOSPHORYLASE ACTIVITY AFTER ADMINISTRATION OF RESERPINE TO RATS
(The phosphorylase assay system contained the following in a total volume of 0·2 ml: 5 mg of tissue, 1% glycogen, 0·016 M glucose-1-phosphate and 0·001 M adenylic acid when required. The mixture was incubated for 5 min at 30 °C.)

Controls (reserpine solvent alone)			Reserpine treated (0·8 mg/kg per day for 7 days)		
μmoles of P liberated without adenylic acid: phosphorylase *a*	μmoles of P liberated with adenylic acid: total phosphorylase *a*+*b*	% phosphorylase *a*: $\frac{a}{a+b} \times 100$	μmoles of P liberated without adenylic acid: phosphorylase *a*	μmoles of P liberated with adenylic acid: total phosphorylase *a*+*b*	% phosphorylase *a*: $\frac{a}{a+b} \times 100$
			Brain		
0·105	0·172	61·0	0·142	0·262	54·2
0·141	0·171	82·5	0·136	0·176	77·3
0·110	0·190	57·9	0·089	0·182	48·9
0·177	0·232	76·3	0·137	0·254	53·9
0·177	0·222	79·7	0·129	0·178	72·5
0·143	0·198	72·2	0·110	0·160	68·8
0·185	0·222	83·3	0·112	0·176	63·6
0·129	0·197	65·5	0·121	0·194	62·4
0·127	0·163	77·9	0·083	0·162	51·2
0·107	0·145	73·8	0·099	0·155	63·9
Mean = 0·140	Mean = 0·191	Mean = 73·0 ± 2·8	Mean = 0·116	Mean = 0·190	Mean = 61·7 ± 2·3
Decrease in % phosphorylase *a* in brain after reserpine: 11; $t = 2{\cdot}9$; $P = 0{\cdot}01$					
			Heart		
0·574	0·755	76·0	0·512	0·784	65·3
0·456	0·651	70·0	0·532	0·776	68·6
0·444	0·615	72·2	0·375	0·693	54·1
0·352	0·516	68·2	0·350	0·527	66·4
0·377	0·565	66·7	0·383	0·572	67·0
0·416	0·661	62·9	0·346	0·612	56·5
0·383	0·535	71·6	0·312	0·601	51·9
0·471	0·626	75·2	0·246	0·418	58·9
Mean = 0·434	Mean = 0·616	Mean = 70·4 ± 1·5	Mean = 0·382	Mean = 0·623	Mean = 61·1 ± 2·3
Decrease in % phosphorylase *a* in heart after reserpine: 9; $t = 3{\cdot}2$; $P < 0{\cdot}01$					

RESULTS

In the summer of 1959 the first experiments were performed on heart and brain employing reserpine. The results are shown in Table 1.

It appeared that reserpine effectively lowered the percentage of phosphorylase *a* in both heart and brain. Noteworthy is the fact that brain, as well as heart, possesses a high initial level of phosphorylase *a*.

In the fall of 1959 and winter of 1960 further experiments were performed with other agents affecting the central nervous system. These were carried out in a randomized fashion and a new series of controls was included. Near completion of the studies it was noted that the new controls for the heart showed values almost exactly those of the earlier controls. However, the brain levels were now several per cent

lower. Time-to-time variations in control observations have been a cause for concern in the past but no definitive explanations have been offered for their occurrence.[9, 12, 18] In any event, if the new brain controls were to be compared to the reserpine-treated animals of the old series, the results would be rendered insignificant; accordingly, a new series of observations on the effect of reserpine was included. The results of all studies in the new series are presented in Table 2.

TABLE 2. PHOSPHORYLASE ACTIVITY AFTER VARIOUS DRUG TREATMENTS

Drug	Mean % phosphorylase *a* ± s.e. Brain	Heart
Control; $n = 16$	64·2 ± 1·8	68·5 ± 1·2
Reserpine PO_4 1·0 mg/kg per day for 7 days; (2nd series); $n = 9$	61·1 ± 2·1	54·7 ± 2·4*
Iproniazid PO_4 25 mg/kg per day and reserpine PO_4 1·0 mg/kg per day given simultaneously for 7 days; $n = 6$	61·0 ± 2·8	55·7 ± 3·3*
Reserpine PO_4 10 mg/kg; after 19–26 hr; $n = 8$	62·0 ± 3·8	54·5 ± 1·8*
Iproniazid PO_4 175 mg/kg given 17–22 hr prior to reserpine PO_4 10 mg/kg; $n = 8$	56·2 ± 3·4	47·0 ± 3·4*
Iproniazid PO_4 100 mg/kg; after 17–22 hr; $n = 6$	69·2 ± 5·0	73·9 ± 3·3
Iproniazid PO_4 250 mg/kg; after 17–22 hs; $n = 5$	66·0 ± 1·5	73·2 ± 2·7
Serotonin creatinine SO_4 20 mg/kg; after ½ hr; $n = 8$	60·1 ± 2·3	71·6 ± 2·6;
Iproniazid PO_4 175 mg/kg given 17–22 hr prior to serotonin creatinine SO_4 20 mg/kg; $n = 8$	61·4 ± 2·1	68·1 ± 2·6
Chlorpromazine 1 hr after 2nd of two doses, 25 mg/kg each, given 17–25 hr apart; $n = 8$	67·2 ± 3·1	69·9 ± 3·3
Pentobarbital 50 mg/kg; after 1 hr; $n = 8$	59·9 ± 2·1	65·8 ± 2·3

* Significant at the 1% level. Test for significance according to: C. W. Dunnett, A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Statist. Ass.* **50**, 1096 (1955).

Once again, chronic reserpine administration was shown to lower the phosphorylase *a* content of the heart, but it appeared that the earlier result with brain was not reproducible. Further, it is seen that single high doses of reserpine also had significant actions in lowering active enzyme in the heart but, here too, the brain effect was not significant.

In regard to other aspects of the study, it was noted that the concurrent administration of or pretreatment with iproniazid did not inhibit the ability of reserpine to lower phosphorylase *a* in the heart. In the rats given reserpine after pretreatment with iproniazid, brain phosphorylase *a* showed a downward trend to a point close to the 5 per cent level of probability. Iproniazid alone produced slight rises which were not significant.

It appeared that serotonin, chlorpromazine and pentobarbital had no effect on the phosphorylase *a* content of heart and brain.

DISCUSSION

One unequivocal positive observation has been noted as a result of the present study. Reserpine significantly lowers the phosphorylase *a* content of the heart of the

rat. This contrasts with the study which reports that reserpine has no effect on phosphorylase *a* in the isolated guinea pig atrium,[15] but correlates well with the observation that reserpinized hearts show an increase of glycogen,[19] a finding which one might expect if glycogenolysis is impaired through an enzyme defect.

Our results with brain are equivocal; the earlier series of experiments indicated significant lowering of phosphorylase *a* in brain upon chronic reserpine administration, but the new series showed no changes. Although a diminution of active enzyme would fit well with the finding that reserpinized animals show an increase in brain glycogen,[20] it should be emphasized that central nervous system effects are noted at a time when phosphorylase *a* is unchanged in the brain of rats treated with single high doses of reserpine; thus, there seems to be no correlation between enzyme activity and the central nervous system action.

The experiments in which iproniazid was given alone were performed on the supposition that a converse result to that obtained with reserpine might be expected. The former agent causes an increase in catecholamine levels in brain.[21] The resultant rise might then lead to increases in active enzyme. However, in regard to brain, it should be noted that, thus far, catecholamines have not been implicated in promoting cyclic nucleotide formation in that tissue.[22]

It was observed that iproniazid, of all agents studied, tended to produce increases in phosphorylase *a* in both heart and brain, but these rises were not significant. This lack of significance would not be surprising, even in the best of situations, since at our control levels of 65 per cent, phosphorylase *a* is already at, or close to, maximum activity.[5] Consequently, any agent which may potentially show increases would be operating against this limitation. Further, a negative result in heart is not unexpected, since rat hearts show only a non-significant 15 per cent rise in catecholamines after iproniazid.[23] Thus, our iproniazid results are inconclusive in that we are uncertain as to whether the lack of significance is real or apparent.

Through a mechanism that is not clear, animals pretreated with iproniazid before reserpine administration are reported to show considerably less of a loss in adrenal, brain[21] and heart[24] catecholamines than if they were treated with reserpine alone. If lowering of phosphorylase *a* is due to impairment of the activation of that enzyme, as a result of reserpine-induced amine depletion, one may test whether iproniazid may prevent the effect of reserpine on phosphorylase *a*.

Our experiments on heart and brain show that iproniazid-pretreatment, or iproniazid given concurrently with reserpine, does not have any effect in preventing the diminution of active enzyme that obtains when reserpine is given alone, particularly in the case of the heart. Unless it is contended that iproniazid does not afford adequate protection, as indeed may not occur in the rat heart,[23] this finding suggests that the effect of reserpine on phosphorylase *a* may not be dependent on catecholamines and, thus, direct or other mechanisms cannot be ruled out.

One interesting observation which in no way resolves our previous dilemma concerning phosphorylase activity in the brain after reserpine administration is that, in the iproniazid-pretreated animals, reserpine produces a downward trend in phosphorylase *a* in brain to an extent that places the result at the borderline of the 5 per cent level of probability. In the heart too, it appears that active enzyme is diminished to an even greater degree after reserpine in the iproniazid-pretreated rats. The significance of this finding is not apparent.

Our study indicates that serotonin, chlorpromazine and pentobarbital have no effect on phosphorylase *a* in brain or heart and, for the present, support the specificity of action of reserpine where that effect is of a positive nature.

It is difficult to assess the therapeutic significance of our findings relating to the definite action of reserpine in lowering phosphorylase *a* in the intact heart. Concern has already been voiced regarding potential dangers with any agent which decreases the amount of the intrinsic catecholamines in the heart.[25] Now, whether it be indirectly through catecholamine depletion or via direct action, we are faced with a significant alteration of an enzyme activity in heart upon reserpine administration. Not until the role of phosphorylase in cardiac metabolism is evaluated can we estimate the importance of the observation.

Addendum. While this work was in progress, an abstract by M. E. Hess, J. Shanfeld and N. Haugaard appeared in *Fed. Proc.* **19**, 108 (1960); in this the action of reserpine in decreasing phosphorylase *a* in the intact rat heart was noted. No data were provided. On the other hand, a still later (July) paper, by S. E. Mayer and N. C. Moran, *J. Pharmacol.* **129**, 271 (1960), indicated that neither reserpine alone nor serotonin had any effect on phosphorylase *a* in the intact dog heart. In June, S. L. Leonard and H. T. Day reported, in *Proc. Soc. Exp. Biol. Med., N. Y.* **104**, 338 (1960), that serotonin had no effect on phosphorylase *a* in rat heart.

Acknowledgements—We are grateful to Dr. Edward Muntwyler, Department of Biochemistry, for the generous donation of the Wistar strain rats used in this study. We should also like to thank Dr. W. E. Scott of Hoffman–LaRoche, Inc. for supplying us with iproniazid ("Marsilid") phosphate and Dr. R. A. Maxwell of Ciba Pharmaceutical Products, Inc. for his contribution of reserpine ("Serpasil") phosphate.

REFERENCES

1. G. T. CORI, *J. Biol. Chem.* **159**, 333 (1945).
2. C. F. CORI, *Enzymes; Units of Biological Structure and Function* (Edited by O. H. GAEBLER) p. 573. Academic Press, New York (1956).
3. E. W. SUTHERLAND, *Ann. N.Y. Acad. Sci.* **54**, 693 (1951).
4. A. A. GREEN and G. T. CORI, *J. Biol. Chem.* **151**, 21 (1943).
5. G. T. CORI and A. A. GREEN, *J. Biol. Chem.* **151**, 31 (1943).
6. E. J. KREBS and E. H. FISCHER, *J. Biol. Chem.* **216**, 113 (1955).
7. T. W. RALL, W. D. WOSILAIT and E. W. SUTHERLAND, *Biochim. Biophys. Acta* **20**, 69 (1956).
8. S. ELLIS, J. MCGILL and H. L. ANDERSON, *Fed. Proc.* **16**, 294 (1957).
9. M. E. HESS and N. HAUGAARD, *J. Pharmacol.* **122**, 169 (1958).
10. W. R. KUKOVETZ, M. E. HESS, J. SHANFELD and N. HAUGAARD, *J. Pharmacol.* **127**, 122 (1959).
11. S. E. MAYER and N. C. MORAN, *Fed. Proc.* **18**, 419 (1959).
12. J. BELFORD and M. R. FEINLEIB, *J. Pharmacol.* **127**, 257 (1959).
13. T. W. RALL and E. W. SUTHERLAND, *J. Biol. Chem.* **232**, 1065 (1958).
14. M. K. PAASONEN and O. KRAYER, *J. Pharmacol.* **123**, 153 (1958).
15. A. GIOTTI, *Arch. Ital. Sci. Farmacol.* **9**, 399 (1959).
16. N. J. GIARMAN, *Yale J. Biol. Med.* **32**, 73 (1959).
17. G. T. CORI and B. ILLINGWORTH, *Biochim. Biophys. Acta* **21**, 105 (1956).
18. S. L. LEONARD, *Endocrinology* **60**, 619 (1957).
19. W. ESTERLUHS. Inaugural Dissertation. Cited by W. ALBRECHT, *Klin. Wschr.* **35**, 588 (1957).
20. W. ALBRECHT, *Klin. Wschr.* **35**, 588 (1957).
21. A. PLETSCHER, *Biochemistry of the Central Nervous System* (Edited by F. BRUCKE) p. 124. Pergamon Press, New York (1959).
22. T. W. RALL and E. W. SUTHERLAND, *Pharmacol. Rev.* **2**, Pt. 2, 464 (1959).
23. A. PLETSCHER, *Experientia* **14**, 73 (1958).
24. A. PEKKARINEN. Cited by W. RAAB, *Cardiologia* **36**, 186 (1960).
25. D. R. WAUD, S. R. KOTTEGODA and O. KRAYER, *J. Pharmacol.* **124**, 340 (1958).