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Inhibitory Effects of Pteroyl Glutamic Acid Preparations.

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Following the suggestion of Ross *et al.*[1] that pteroyl glutamic acid (PGA)† might exert an inhibitory effect on the glutamic acid metabolism of brain, experiments have been performed on the effect of PGA on the respiration of brain cell suspensions *in vitro*. The experiments were originally planned to test the hypothesis that PGA may act as a metabolic competitor of glutamate or other related substrates. At first, glutamate and PGA were used in approximately equimolar concentrations, 0.005 molar. Later, PGA effects were also studied in concentrations of 0.001 molar and 0.025 molar, keeping substrate concentrations 0.005 molar.

It is not to be inferred that such high concentrations of PGA bear any relation whatever to therapeutic concentrations, but only to the information that might be gained regarding their competitive action with glutamate and other substrates.

It may be noted in this connection that the glutamate concentration in human plasma is normally about 1 mg per 100 cc,[2] whereas the PGA concentration has been reported by Denko *et al.*[3] to be only 2 μg per 100 cc. On the basis of these data, the molar concentration of PGA in plasma is about 1/1000 that of glutamate.

As will appear below, inhibiting effects when present seem to be nonspecific and by no means restricted to the inhibition of respiration when glutamate is the added substrate. Furthermore, pure pteroyl glutamic acid seems to be without inhibitory effects even at a concentration of 0.025 molar.

Methods. The oxygen consumption of cell suspensions prepared from the brains of Wistar strain white rats have been measured in the Warburg apparatus at 37°C. These were prepared in a loose fitting homogenizer of the type devised by Potter and Elvehjem[4] that was driven at 100-200 r.p.m. Eighty mg fresh weight of tissue were pipetted into each flask. The gas phase was air and the shaking rate was 105 strokes per minute with a stroke displacement of 3 cm. The suspending medium was NaCl 0.136 M, KCl 0.004 M, $MgCl_2$ 0.0005 M, and phosphate buffer 0.0075 M (pH 7.4). Calcium was deliberately omitted from the incubating medium to avoid the danger of combination with or precipitation of PGA. Its omission was found to have no demonstrable effect on the respiration results.

The substrates used were: glutamate, pyruvate, lactate, succinate, and α-ketoglutarate, each at a concentration of 0.005 molar. Through the kindness of Dr. Y. SubbaRow and his associates at the Lederle Laboratories, 5 preparations of commercial PGA, a preparation of freshly recrystallized $(PGA)_R$, and a sample of the photofission product, 2-amino-4-hydroxy-6-formylpteridine, or pteridine aldehyde, were made available to us for testing. In addition, a sample of PGA was recrystallized in our laboratory.

The solutions of substrates and of PGA were adjusted to pH 7.2-7.5 before use. The elapsed time between the sacrifice of the rat and the first readings of the manometers varied from 20-30 minutes. Manometer readings were taken at 10-15 minute intervals for

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1 Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, **3**, 68.

† The abbreviation PGA will be used to denote pteroyl glutamic acid preparations kindly supplied us by Lederle Laboratories. $(PGA)_R$ will be used to denote freshly recrystallized PGA.

2 Bessman, S. P., Magnes, J., Schwerin, P., and Waelsch, H., *J. Biol. Chem.*, 1948, **175**, 817.

3 Denko, C. W., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

4 Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

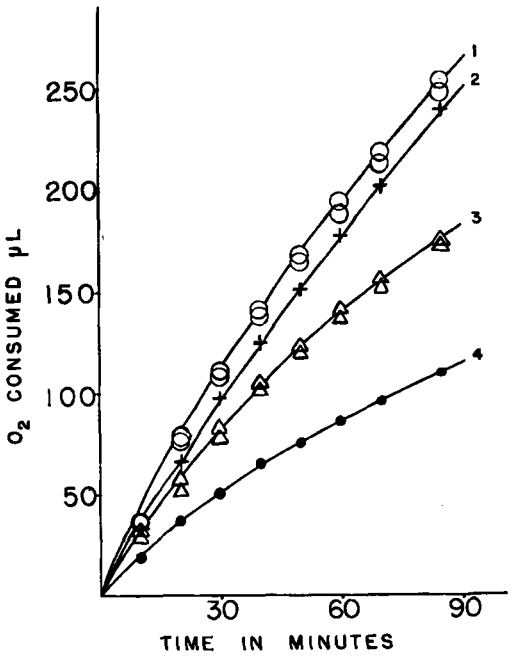

FIG. 1.

Experiment No. 50, comparison of the effect of PGA and recrystallized PGA, $(PGA)_R$, on the oxygen uptake of rat brain suspension in the presence of pyruvate. 1. Pyruvate 0.005 M with $(PGA)_R$ 0.025 M. 2. Pyruvate 0.005 M. 3. Pyruvate 0.005 M with PGA 0.025 M. 4. No substrate added. Paired circles, curve 1, and paired triangles, curve 3, denote duplicate determinations.

90 minutes and converted to microliters (μL) of oxygen consumed. Results are reported in terms of Q_{O_2} (μL per mg dry tissue per hour) for the 60-90 minute portion of each experiment. This period was chosen in order to increase the opportunity for any influence of added PGA preparations to become evident.

Results and Comment. The results of a typical experiment are shown in Fig. 1. This illustrates the doubling of the respiration rate produced by the presence of 0.005 molar pyruvate (compare curves 2 and 4); the absence of inhibitory effects by recrystallized pteroyl glutamate $(PGA)_R$, 0.025 molar, and the considerable inhibitory effect produced by a commercial preparation of PGA, 0.025 molar.

In Table I are presented the control Q_{O_2} values observed in the presence of various substrates, and the degree of inhibition produced by the addition of commercial PGA preparations in concentrations of 0.001 M, 0.005 M, and 0.025 M, respectively. It will be noted that some inhibition was produced in the presence of all substrates, or, indeed, in the absence of any added substrate, when the PGA preparation was in a concentration of 0.025 molar. The greatest inhibition was observed with pyruvate, lactate, and succinate; the least with α-ketoglutarate, glutamate, or no added substrate. No significant inhibition was observed when PGA was in a concentration of 0.001 molar; at PGA concentrations of 0.005 molar, the respiration with pyruvate as substrate was decreased 30%; with other substrates, it was not significantly altered.

In view of the absence of inhibitory effects of PGA in a concentration of 0.001 molar and in view of the non-specific nature of the inhibition encountered at PGA concentrations of 0.025 molar, it was concluded that no specific competition of PGA with glutamate was indicated by the present experiments.

Since the respiration with pyruvate seemed to be the most sensitive to additions of PGA, this substrate was used in subsequent experiments to determine (a) whether freshly recrystallized $(PGA)_R$ exerted any inhibitory effect and (b) whether breakdown products of PGA could account for the inhibition. The results of these experiments are presented in Table II.

It was found that freshly recrystallized $(PGA)_R$ exerted no significant inhibitory effect even at the high concentration of 0.025 molar, whereas experiments with commercial PGA run at the same time showed between 40 and 50 percent inhibition of respiration. From this it may be concluded that the inhibition of respiration encountered in the previous experiments was not due to the pteroyl glutamic acid present. This is in agreement with the results obtained by Franklin *et al.*[5]

Efforts were then directed to a study of the substances producing the inhibitory effect.

[5] Franklin, A. L., Regan, M., Lewis, D., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 523.

[6] Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, **172**, 349; Kalckar, H. M., Kjeldgaard, N. V., and Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

TABLE I.

Effect of PGA Preparations on Oxygen Consumption of Rat Brain Suspensions. $Qo_2 = \mu L\ O_2$ consumed per hour per mg dry tissue for the 60-90 minute period of observation. Concentration of all substrates = 0.005 M.

Substrate	Control Qo_2	% inhibition PGA .001 M	PGA .005 M	PGA .025 M
Pyruvate	7.9	9	30	53
Lactate	4.7	—	—	45
Succinate	4.4	0	11	41
α-Ketoglutarate	3.8	5	13	21
Glutamate	3.8	3	16	21
None	2.3	—	—	26

TABLE II.

Comparison of the Effect of $(PGA)_R$, PGA and Pteridine Aldehyde on the Qo_2 of Rat Brain Suspensions for the 60-90 Minute Period. Substrate = 0.005 molar pyruvate. All experiments represent averages of duplicate or triplicate determinations. The concentrations of $(PGA)_R$ and PGA were 0.025 M; the concentration of pteridine aldehyde was 0.00026 M.

Exp. No.	Control Qo_2	% inhibition $(PGA)_R$, 0.025 M	PGA, 0.025 M
50	8.1	4	40
55	8.1	13	52
		Pteridine aldehyde	PGA, 0.025 M
61	8.8	32	—
62	7.4	38	59
63	7.7	27	47

Kalckar *et al.*[6] have found that the aldehyde photofission product of PGA, 2-amino-4-hydroxy-6-formylpteridine or pteridine aldehyde, is toxic to xanthine oxidase, xanthopterin oxidase, and quinine oxidase; and Lowry[7] has found that this substance is toxic to pteridine oxidase. Experiments were, therefore, carried out using pteridine aldehyde in a saturated solution. (This compound is only slightly soluble at pH 7.4, and in our experiments had a concentration of 5 mg per 100 cc or 0.00026 molar.) The results of these experiments are also presented in Table II. It was found that with pyruvate as substrate, the pteridine aldehyde in very low concentration inhibited the oxygen consumption of rat brain suspensions about 30%. Parallel experiments using an uncrystallized preparation of PGA caused an inhibition of about 50%. It would appear from these observations that, since the pteridine aldehyde is a breakdown product of PGA, it may well account in large measure for the inhibitory effects observed with commercial PGA preparations. Whether there are also additional inhibitory substances present must await further work.

Summary. 1. Freshly recrystallized pteroyl glutamic acid $(PGA)_R$ even in very high concentrations exerts no significant inhibitory effect on the metabolism of brain cell suspensions.

2. PGA preparations, not freshly recrystallized, have a marked inhibitory action in high concentrations (25 millimolar). In lower concentrations (1 millimolar), the inhibition is negligible.

3. The inhibitory action with pyruvate as substrate can be largely accounted for by the photofission product of PGA (2-amino-4-hydroxy-6-formyl-pteridine).

[7] Lowry, O. H., personal communication.