The author is very grateful to Professor A. Wormall, F.R.S., for his encouragement, and to Miss A. Routledge for her valued technical assistance. The work was supported by a grant to St Bartholomew's Hospital from the British Empire Cancer Campaign.

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Uptake of Pyrithiamine by Tissue of Rats

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(*Received* 13 *December* 1960)

Although the thiamine and thiamine diphosphate content of the tissues of animals treated with pyrithiamine, 1-[(4-amino-2-methyl)pyrimid-5-ylmethyl]-2-methyl-3-(β-hydroxyethyl)pyrimidinium bromide hydrobromide, has been studied, no data are available regarding the pyrithiamine content that may be related to thiamine levels, and that might clarify the mechanism producing avitaminosis B_1 in animals treated with pyrithiamine. Administration of pyrithiamine causes a decrease of the total thiamine and thiamine diphosphate content of all the tissues, particularly of the brain, in mice and rats (De Caro, Rindi & Grana, 1954; De Caro, Rindi, Perri & Ferrari, 1956*a*, *b*, 1958; De Caro, Rindi & Ferrari, 1958; Koedam & Steyn-Parvè, 1959), and in pigeons (Koedam, Steyn-Parvè & van Rheenen, 1956; Koedam, 1958). The diminution in the brain is both marked and early, whereas in the alimentary thiamine deficiency the brain more firmly preserves its thiamine content (Salcedo, Najjar, Holt & Hultzer, 1948; Rindi, Ferrari & Perri, 1954).

The present paper describes a microfluorimetric method for determining pyrithiamine in rat tissues and reports the results obtained in animals treated with pyrithiamine, with and without thiamine.

EXPERIMENTAL

Materials. Adsorbent charcoal was prepared by treatment of activated charcoal (50SL, produced by Italian C.E.C.A., Milan) with cholesteryl stearate (British Drug Houses Ltd.), as described by Siliprandi & Siliprandi (1954). Columns were prepared according to Rindi & de Giuseppe (1961). Amberlite IRC-50 resin (British Drug Houses Ltd., CG-50) was utilized in a carboxylic form after treatment with 2N-HCl, followed by washing with water until disappearance of the Cl^- reaction. Pyrithiamine was a commercial sample of Neopyrithiamine hydrobromide purchased from the California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A. The mono- and di-phosphate were prepared by the method of Viscontini, Bonetti & Karrer (1951) and purified on ion-exchange resin. Cellulose powder (Whatman, ash-free cellulose) was washed with 50% (v/v) ethanol, filtered on a Buchner funnel, thoroughly rinsed with water and dried.

Determination of pyrithiamine. The rapidly excised tissue (liver, brain, kidney, 3–5 g.; muscle, 9–11 g.) was weighed and homogenized in 2·5 vol. of 0·5M-$HClO_4$ in a MSE homogenizer. After centrifuging (3500 *g*), the clear supernatant fluid was separated and the residue again homogenized in 1 vol. of 0·5M-$HClO_4$ and centrifuged. The combined supernatant fluids were neutralized (pH 6·8–6·9) with 4M-KOH. After 20 min. at 0°, the precipitated $KClO_4$ was removed by centrifuging in a refrigerated centrifuge. To the clear extract, at room temperature and adjusted to pH 4·5 with 50% (v/v) acetic acid, 1 ml. of a freshly prepared aqueous suspension (100 mg./ml.) of Taka-diastase (Parke Davis and Co. Ltd.) was added. The mixture was incubated overnight at 37°. The solution, adjusted to pH 6·8–6·9 with NaOH, was then passed through a column (1·2 cm. diam.) of charcoal–cellulose powder (1:5); 200 mg. of charcoal for liver, brain and kidney, 300 mg. for muscle. The flow rate was 6–8 drops/min. The column was washed with 30 ml. of water and eluted with 25 ml. of 10% (v/v) propan-1-ol (Rindi & de Giuseppe, 1961). The eluate was passed through a column (3 cm. × 0·9 cm.) of Amberlite IRC-50. The flow rate was 10–12 drops/min. The column was washed with 20 ml. of water and eluted with about 25 ml. of 0·1N-HCl. The eluate was made up to 25 ml. with the eluent, if necessary. Solid KCl (400 mg.) was dissolved in each of two samples of 1–2 ml. of eluate, adjusted to 2 ml. with water, in stoppered centrifuge tubes; then 0·5 ml. of 10N-NaOH was added to each. The samples were incubated for 60 min. at 37° to destroy the thiamine and 0·5 ml. of 10N-NaOH was added to one tube (B) and 0·5 ml. of 0·20% (w/v) $K_3Fe(CN)_6$ in

10 N-NaOH (cf. Sealock & White, 1949) to the other (A). After 4 min. the solutions were extracted by shaking for 90 sec. with 5 ml. of *iso*butanol. The aqueous layer was removed after centrifuging, and the *iso*butanol was dehydrated by addition of 0·5 g. of anhydrous Na_2SO_4 and centrifuged. The fluorimetric measurement was performed on 1 ml. of the dried *iso*butanol layer in a model A Farrand microfluorimeter, and compared with a quinine standard (20 μmg./ml. of 0·1 N-H_2SO_4). The difference between the fluorescence values of A and B gave the fluorescence corresponding to pyrithiamine. A calibration curve, which was linear over the range 0–9 μg., was obtained from an aqueous solution of pyrithiamine treated as above, beginning with chromatography on Amberlite resin. The addition of 3 μg. of thiamine caused no significant interference. Under the above conditions a fluorescence value was obtained with tissues from rats which had not been treated with pyrithiamine. These values have been subtracted from the values obtained for tissues of treated rats. The values corresponded to a pyrithiamine value (μg./g. of wet tissue) of about 0·27 in brain, 0·97 in kidney, 1·76 in liver and 0·67 in muscle.

Phosphorylated pyrithiamine. The $HClO_4$ extract of the tissue, after removal of excess of $HClO_4$, was divided into two parts. Fraction A, brought to pH 5·2 with 50% (v/v) acetic acid, was treated with 10 mg. of freeze-dried acid phosphatase prepared from human prostate according to Vescia & Testi (1958). This fraction was then incubated for 8 hr. at 37°, while fraction B, brought to pH 5·2, was stored for the same time in a refrigerator. Both fractions were successively brought to pH 6·8 and treated as described for the determination of pyrithiamine. The intensity of fluorescence of fraction A corresponded to 'total pyrithiamine' (free + phosphorylated) and that of fraction B to 'free pyrithiamine'. Pyrithiamine mono- and di-phosphate give a fluorescent oxidation product insoluble in *iso*butanol.

Animals. Male albino rats, weighing 85–100 g., placed in single small cages, were reared on a synthetic thiamine-deficient diet, consisting of (%): washed, fat-free casein, 20; wheat starch, 63; olive oil, 10; cod-liver oil, 2; Osborne & Mendel's (1912) salt mixture, 5. The diet was supplemented with suitable amounts of all the B vitamins (except thiamine), administered daily by mouth in aqueous solution.

RESULTS

Table 1 summarizes the results obtained in experiments on rat tissues removed 24 hr. after the intraperitoneal injection of 1 mg. of pyrithiamine, to which various amounts of pyrithiamine were added during perchloric acid extraction. The recovery ranged from 96·8 to 102·5%

Tables 2 and 3 show average changes of pyrithiamine content of tissues found after administration

Table 1. *Recovery of pyrithiamine added to some rat tissues*

Tissues were taken from rats 24 hr. after the intraperitoneal injection of 1 mg. of pyrithiamine. Results are given as means ± S.E. for 4 determinations.

	Pyrithiamine (μg./g. of wet tissue)			Recovery (%)
	Present	Added	Found	
Brain	1·42 ± 0·16	2·33 ± 0·44	3·70 ± 0·44	97·6 ± 4·03
Kidney	2·41 ± 0·33	2·57 ± 0·32	4·86 ± 0·27	97·5 ± 4·57
Liver	4·25 ± 0·22	2·44 ± 0·14	6·87 ± 0·36	102·5 ± 4·83
Muscle	0·51 ± 0·02	0·76 ± 0·06	1·24 ± 0·10	96·8 ± 3·38

Table 2. *Pyrithiamine content of rat tissues after a single intraperitoneal injection of* 1 *mg. of pyrithiamine*

The dose was given in 0·2 ml. of 0·9% NaCl. Each value is the average of 7 determinations ± S.E.

	Pyrithiamine (μg./g. of wet tissue)				
Days after dose ...	1	3	5	9	12
Brain	1·13 ± 0·12	1·41 ± 0·21	2·13 ± 0·12	2·25 ± 0·23	2·77 ± 0·17
Kidney	2·65 ± 0·38	1·57 ± 0·28	1·18 ± 0·15	0·98 ± 0·40	0·71 ± 0·24
Liver	7·74 ± 0·78	5·13 ± 0·74	4·75 ± 0·65	2·58 ± 0·62	1·07 ± 0·29
Muscle	1·45 ± 0·15	0·79 ± 0·18	0·44 ± 0·05	0·46 ± 0·08	0·27 ± 0·07

Table 3. *Pyrithiamine content of tissues in rats given a daily oral dose of* 33 μg. *of thiamine* + 210 μg. *of pyrithiamine*

Each value is the average of 6 determinations ± S.E.

	Pyrithiamine (μg./g. of wet tissue)		
Day of treatment ...	5	12	20
Brain	0·91 ± 0·09	1·62 ± 0·07	3·51 ± 0·20
Kidney	1·54 ± 0·19	2·49 ± 0·19	6·49 ± 0·73
Liver	2·52 ± 0·26	4·63 ± 0·48	9·52 ± 0·80
Muscle	0·44 ± 0·12	0·86 ± 0·08	1·77 ± 0·19

of pyrithiamine. The livers of rats which had been injected 24 hr. previously with 1 mg. of pyrithiamine were examined for phosphorylated pyrithiamine. The mean values ± S.E. for 7 determinations of pyrithiamine before and after phosphatase treatment were 0·03 ± 0·01 and 4·37 ± 0·34 g./g. of wet tissue respectively.

DISCUSSION

Content of pyrithiamine in tissues. Twenty-four hours after the intraperitoneal injection of a single dose (1 mg.) of pyrithiamine, the amount found in the tissues was approximately that of the thiamine usually present in the same tissue, except in the brain where the pyrithiamine content was notably less. If the animals did not receive thiamine (Table 2), the pyrithiamine content decreased in liver, kidney and skeletal muscle, but increased in brain, where, at the end of 12 days, it reached a concentration very near to that of thiamine usually present in normal rats.

The considerable thiamine needs of the brain, an organ with predominantly carbohydrate metabolism, are probably normally met by uptake of thiamine from the liver. When, however, pyrithiamine is present in excess of thiamine in the liver, the brain takes up the pyrithiamine. As a consequence, the content of pyrithiamine in brain greatly increases (Table 2), replacing thiamine in the tissue and inducing the early appearance of neuromuscular signs of beri-beri in the rats. This is a peculiar feature of the pyrithiamine treatment, in contrast with alimentary thiamine deficiency. Under similar experimental conditions, De Caro, Rindi, Perri & Ferrari (1958) found that intraperitoneal injection of pyrithiamine to rats or mice resulted in a marked early decrease of the total thiamine content of the brain, the organ in which we have found the highest pyrithiamine concentration. The present results support the view that pyrithiamine induces a thiamine deficiency almost exclusively in the brain.

The daily administration, by mouth, of a mixture of pyrithiamine and thiamine, in suitable proportions, resulted in a continuous and gradual increase of the pyrithiamine content in all the tissues examined (Table 3). De Caro, Rindi, Perri & Ferrari (1958) showed that under the same conditions there was a decrease of the total thiamine concentration of the tissues, quite comparable with that found after administration of pyrithiamine alone, but requiring a far longer period of treatment before being reached, perhaps as a consequence of the daily intake of thiamine.

Form of pyrithiamine in the tissues. The results obtained after treatment of liver extract with phosphatase suggest that, 24 hr. after the intraperitoneal injection of a rat with 1 mg. of pyrithiamine, the liver contains pyrithiamine which is practically all in a phosphorylated form. Pyrithiamine *in vitro* inhibits the hepatic (Eich & Cerecedo, 1954) and intestinal (Cerecedo, Eich & Bresnick, 1954) thiaminokinase. It seems likely that this enzyme could phosphorylate pyrithiamine, especially when present in the amount found in the liver 24 hr. after its injection.

SUMMARY

1. A microfluorimetric method for the determination of pyrithiamine in animal tissues is described.

2. The intraperitoneal injection of 1 mg. of pyrithiamine in rats, reared on a thiamine-deficient diet, caused an initial increase of the pyrithiamine content in the liver, muscle, kidney and brain, followed by a steady decrease in all the tissues except the brain, where a final increase of up to 200 % was found.

3. The daily oral administration of 210 μg. of pyrithiamine together with 33 μg. of thiamine, for 20 days, induced a gradual increase of the pyrithiamine content in all the tissues examined.

4. After a single intraperitoneal injection of 1 mg., pyrithiamine was found 24 hr. later in the liver almost all in a phosphorylated form.

We are deeply grateful to the Research Corporation, New York, for the generous gift of a Farrand fluorimeter.

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