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The B-vitamin group and the activity of hepatic microsomal mixed-function oxidases of the growing Wistar rat

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1. Male Wistar rats were given isoenergetic, semi-synthetic diets deficient in thiamin, riboflavin, pyridoxine or all the B-vitamins.

2. In rats given these deficient diets the 'sleeping time' induced with pentobarbital (PB) and the 'paralysis time' with zoxazolamine (Zz) were prolonged.

3. The tolerance effect against both drugs was nearly independent of the levels of B-vitamins in the diets.

4. In preparations from vitamin-B deficient animals the activities of the following hepatic microsomal enzymes were reduced: the aliphatic hydroxylase of PB, the aromatic hydroxylases of aniline (*EC* 1.14.14.1) and of Zz, the *N*-demethylase of aminopyrine, the UDP glucuronyltransferase (*EC* 2.4.1.17) of *p*-nitrophenol. The reactions most influenced were those of 'type-1' substrates, particularly those involving the hydroxylases.

5. The effects observed were caused mainly by deficiency of riboflavin and to a lesser extent of thiamin or pyridoxine.

The intake of nutrients and of 'foreign' chemicals (non-nutrients), for example drugs or pollutants, represent two major groups of factors which are influencing health. The effect of the intake of 'foreign' chemicals on the metabolism of an organism depends not only on its amount and its type, but also on the genetic characteristics and the physiologic condition and nutritional status of the organisms.

Any deviation from the normal composition of the diet may force the organism to adapt its metabolism. This can involve alterations in the metabolism and action of non-nutrients. Results of several investigations have provided evidence of such interactions between nutrients and non-nutrients (Oltersdorf, Miltenberger & Cremer, 1977). The possibilities for interaction between individual nutrient deficiencies and intakes of particular 'foreign' chemicals are very complex; even more complex are the possible multi-sided interactions. Obviously it should not be the aim of investigations to close arbitrary gaps of the matrix of interactions, but rather to test the influence of a different nutrient intake on specific metabolic reactions which are commonly involved in the metabolism of 'foreign' chemicals.

Lipophilic 'foreign' chemicals, which include most of the drugs, are catabolized by the hepatic microsomal enzymes, a multi-enzyme complex. This enzyme complex is rather non-specific with respect to its substrates, and uses cytochrome P_{450} as terminal oxidase (Estabrook, 1971; Gilette, Davis & Sasame, 1972).

There are two different types of cytochrome P_{450}-substrate complexes which can be differentiated by their absorption spectra (Remmer, Schenkman, Estabrook, Sasame, Gilette, Narashimhulu, Cooper & Rosenthal, 1966; Schenkman, Remmer & Estabrook, 1967): 'type-1' substrate (e.g. hexobarbital), 'type-2' substrate (e.g. aniline).

The activity of these enzymes (drug-metabolizing enzymes; DME) is dependent on many factors (Vesell, 1972; Dickerson & Walker, 1974). Their activity can be increased by repeated administration of several chemicals, but mainly by 'type-1' substrates. This induction of the microsomal enzymes is the main factor involved in the increasing tolerance to foreign chemicals (Conney, Miller & Miller, 1956; Remmer, 1959; Wattenberg, 1973).

The nutritional status of an organism has a marked influence on the DME systems (Campbell & Hayes, 1974). The role of vitamin B deficiencies in the drug metabolism has not as yet been sufficiently investigated. The effect of a general deficiency of B-vitamins was tested only by measuring the drug action in vivo (e.g. sleeping time). The same holds true for pyridoxine deficiency. The influence of the B-vitamins on the process of increasing tolerance to drugs has not yet been examined (Campbell & Hayes, 1974; Oltersdorf *et al.* 1977). Regarding the influence of thiamin and riboflavin on the drug metabolism there are some conflicting results. Levy & Di Palma (1953) could not show any influence of thiamin deficiency on the sleeping times after administration of thiopental and hexobarbital. However, the experiments of Kalyanpur, Naik & Sheth (1968) revealed that thiamin pretreatment increased the sleeping time after pentobarbital administration, but not in the case of hexobarbital. The influence of thiamin on DME was dependent on the substrate, e.g. the metabolism of aniline and of heptachlor was accelerated in thiamin deficiency, whereas that of hexobarbital was decreased (Wade, Greene, Ciordia, Meadows & Caster, 1969). Riboflavin deficiency caused a prolongation of the sleeping times after thiopental application (Levy & Di Palma, 1953) and after hexobarbital application (Catz, Juchau & Yaffe, 1970). The rate of hydroxylation of aniline and acetanilide was markedly reduced in liver preparations of riboflavin-deficient rats (Patel & Pawar, 1973, 1974); whereas in similar experiments with mice no influence of riboflavin deficiency on the activities of these enzymes could be discovered (Catz *et al.* 1970).

The aim of this present study was to investigate with rats the influence of deficiencies of the vitamin B-complex and of individual deficiencies of thiamin, riboflavin and pyridoxine on the action, the induction and the metabolism of 'model' drugs, i.e. the 'type-1' substrate pentobarbital (PB) and the 'type-2' substrate zoxazolamin (Zz). Their action was measured as 'sleeping time' and 'paralysis time', respectively. These measurements give an indication of their metabolism and thus of the activity of the DME system. Additionally the drug effects on the body temperature were determined. The in vivo results were compared with in vitro determinations of the activity of mixed-function oxidases in liver homogenates. The 'model' drugs were administered repeatedly in order to induce maximum tolerance against them. Preliminary experiments showed that four daily doses were sufficient.

MATERIALS AND METHODS

Inbred male Wistar rats (Zentralinstitut fuer Versuchstierkunde, Hannover, W. Germany) were used. Their age was 5 weeks and they weighed 80–90 g. They were given Altromin-oatmeal chow (Altromin GmbH, D 4937 Lage (Lippe), W. Germany) for 3 d before being allocated to the test diets. The isoenergetic, semi-synthetic diets differed only in the content of the B-vitamins; their composition is shown in Table 1. The diet with the multiple deficiency of B-vitamins fed to group DG was deficient also in several vitamins which are regarded as non-essential for rats. Nevertheless some of these vitamins might have had an influence on the tested parameters. Therefore a comparison of the results of the isolated vitamin B deficiencies with those of this group (DG) could indicate whether it would be worth while to test further isolated deficiencies. The intake of B-vitamins in group DG rats was approximately 50% of the requirement; the diets deficient in thiamin (group DT), riboflavin (group DR) and pyridoxine (group DP) similarly provided about 50% of the requirement, but all other vitamins were added in liberal amounts. All rats received the same restricted amount of food, except the *ad lib.*-fed control group (group CA). The amount of diet was adjusted to 11–12 g/d per rat which allowed all rats to ingest the food completely (equal feeding). The rats had free access to water and were maintained separately in wire-bottomed cages in an air-conditioned room (22°, 50% relative humidity, normal daylight hours).

Table 1. *Composition of the experimental diets* given to groups of rats*

Ingredient	Group				
	CA and CE	DG	DT	DR	DP
Vitamin-free casein (g)	250·0	250·0	250·0	250·0	250·0
Methionine (g)	2·0	2·0	2·0	2·0	2·0
Saccharose (g)	550·0	550·0	550·0	550·0	550·0
Rice starch (g)	86·0	86·0	86·0	86·0	86·0
Lard (g)	62·5	62·5	62·5	62·5	62·5
Maize oil (g)	7·5	7·5	7·5	7·5	7·5
Retinyl acetate (g)	0·1	0·1	0·1	0·1	0·1
Ergocalciferol (mg)	5·5	5·5	5·5	5·5	5·5
α-Tocopheryl acetate (mg)	114·5	114·5	114·5	114·5	114·5
Choline chloride (g)	1·7	1·7	1·7	1·7	1·7
Salt mix† (g)	40·0	40·0	40·0	40·0	40·0
Thiamin (mg)	22·0	1·0	1·0	22·0	22·0
Riboflavin (mg)	25·0	1·5	25·0	1·5	25·0
Pyridoxine (mg)	25·0	1·5	25·0	25·0	1·5
Calcium pantothenate (mg)	62·5	5·0	62·5	62·5	62·5
myo-Inositol (mg)	112·5	20·0	112·5	112·5	112·5
Cyanocobalamin (μg)	30·0	4·0	30·0	30·0	30·0
p-Aminobenzoic acid (mg)	112·5	0	112·5	112·5	112·5
Nicotinic acid (mg)	100·0	0	100·0	100·0	100·0
Biotin (mg)	0·5	0	0·5	0·5	0·5
Pteroylmonoglutamic acid (mg)	2·0	0	2·0	2·0	2·0
Ascorbic acid (g)	1·0	0	1·0	1·0	1·0
Menaphthone (mg)	50·0	0	50·0	50·0	50·0

* Based on diets of Hoetzel (1962) and Moore & Yontz (1969).
† *US Pharmacopoeia* XIII (1947), p. 721.

After 12 weeks on the deficient diets, growth of the rats decreased significantly. The rats in each of the differently-fed groups (forty rats in each group) were then subdivided further (matched for body-weight) into 3 subgroups of eight to thirteen rats. Subgroup 1 (control) received intraperitoneally (i.p.) physiological saline (9 g sodium chloride/l), subgroup 2 received i.p. 30 mg PB/kg body-weight (as the sodium salt of PB, Nembutal®; Deutsche Abott GmbH, Ingelheim, W. Germany), and subgroup 3 received i.p. 60 mg Zz/kg body-weight (Flexin®; McNeil Laboratories, Philadelphia, Pennsylvania, USA). The injections (Schneider & Schneider, 1970) were given on four successive days in the morning before the rats were fed. The drugs were dissolved in physiological saline (Conney, Trousof & Burns, 1960; Kato, Takayanagi & Oshima, 1969) and injected at a dose of 5 ml/kg body-weight.

The animals were killed immediately on awakening after the fourth injection by opening the thorax under anaesthesia with diethyl ether.

The food intake of the animals was recorded daily, and the body-weight twice weekly. The action of the drugs was measured by recording the 'PB sleeping time', the 'Zz paralysis time' (Vesell, 1968), and the rectal body temperature (measured with the apparatus Tastomed PZ, Braun Electronic GmbH, Frankfurt/M., W. Germany) during drug action.

Heparinized blood was collected from the opened thorax for the determinations of erythrocyte transketolase (*EC* 2.2.1.1; ETK) (Schouten, van Eps & Boudier, 1964; modified by Hoffmann-La Roche, personal communication), erythrocyte glutathione reductase (*EC* 1.6.4.2; EGR) (Glatzle, Weiser, Weber & Wiss, 1973), erythrocyte aspartate: 2-oxoglutarateaminotransferase (*EC* 2.6.1.1; EGOT) (Raica & Sauberlich, 1964; modified by Hoffmann-La Roche, personal communication), PB (Brodie, Burns, Mark, Lief, Bernstein & Papper, 1953) and Zz (Conney *et al.* 1960).

Table 2. *Activities of erythrocyte enzymes of rats given a control diet ad lib. (group CA) and equal-fed (group CE), and given diets deficient in several B-vitamins (group DG), thiamin (group DT), riboflavin (group DR), and pyridoxine (group DP)**

(Mean values and standard deviations)

Group	Erythrocyte transketolase† (*EC* 2.2.1.1)		Erythrocyte glutathione reductase‡ (*EC* 1.6.4.2)		Erythrocyte aspartate: 2-oxoglutarate aminotransferase§ (*EC* 2.6.1.1)	
	n	Mean SD	*n*	Mean SD	*n*	Mean SD
CA	16	118 20	—		—	
CE	15	122 31	12	1·26 0·09	12	1·03 0·04
DG	12	16 7	22	1·94 0·21	—	
DT	9	21 18	—		—	
DR	—		21	1·89 0·15	—	
DP	—		—		17	1·53 0·17

* For details of diets, see Table 1.

† μmol Sedoheptulose-5-phosphate/min per l erythrocyte suspension (with packed cell volume 0·35) at 37°.

‡ Activation index; enzyme activity in the presence of FAD: activity in the absence of FAD.

§ Activation index; enzyme activity in the presence of pyridoxal-5-phosphate: activity in the absence of pyridoxal-5-phosphate.

Livers and brains were removed, washed in ice-cold saline, dried on blotting paper and stored at 0–4°.

The liver was sliced and homogenized with 4 vol. ice-cold 0·154 M-potassium chloride in a Potter-Elvehjem homogenizer. The homogenizer was operated at 1500 rev./min for 1 min and cooled with ice (von Jagow, Kampffmeyer & Kiese, 1965; Benoehr, Franz & Krisch, 1966). This was done in a cooled room at 0–4°. The homogenate was centrifuged for 30 min at 9000 *g*, or 10 min at 1470 *g* for the estimation of glucuronyltransferase using a refrigerated centrifuge (Sorvall model RC 2-B, rotor SS 34; I. Sorvall, Inc., Newtown, Connecticut, USA). The supernatant fractions were used in subsequent assays for microsomal DME.

Protein content of livers and homogenates was determined according to Lowry, Rosebrough, Farr & Randall (1951). The activities of the following DME were tested: UDP glucuronyltransferase (*EC* 2.4.1.17) with *p*-nitrophenol (*p*-Np) as substrate (Mulder, 1970), aliphatic hydroxylase of PB ('type-1' substrate) (Kato *et al.* 1969), *N*-demethylase of aminopyrine ('type-1' substrate) (Gram, Wilson & Fouts, 1968), aromatic hydroxylases of Zz ('type-2' substrate) (Conney *et al.* 1960) and of aniline ('type-2' substrate) (Guarino, Gram, Gigon, Greene & Gilette, 1969).

In brain homogenates the concentrations of PB (Brodie *et al.* 1953) and Zz (Conney *et al.* 1960) were estimated.

All determinations were done within 24 h after biopsy collection. The results were statistically evaluated by Student's *t* test.

RESULTS

In vitamin B deficiencies the rats became anorexic, their food intake (g) decreased from 17·6 ± 1·0 in CA rats to 11–12 in the rats given the deficient diets.

The body-weight (mean ± SE; g) of the rats was highest in the CA group (*ad lib.*-fed group) (443 ± 77) and very similar in the equal-fed groups (CE 329 ± 57, DG 294 ± 33, DT 301 ± 41, DR 337 ± 22, DP 324 ± 33).

Table 3. *'Sleeping time' after pentobarbital injections (30 mg/kg intraperitoneally) on four successive days of rats given a control diet ad lib. (group CA) and equal-fed (group CE), and given diets deficient in several B-vitamins (group DG), thiamin (group DT), riboflavin (group DR), and pyridoxine (group DP)**

(Mean values and standard deviations)

Period after administration of drug (d) ...		'Sleeping time' (min)								
		1		2		3		4		
Group	*n*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	PTI
CA	9	40	2	34	2	31	1	30	1	133
CE	12	51	2	37	3	34	2	31	1	162
DG	9	65	6	53	4	47	3	44	3	146
DT	13	57	3	41	3	39	3	36	3	158
DR	12	63	4	47	4	43	3	39	3	155
DP	13	57	3	42	3	39	4	37	4	154

PTI, per cent tolerance index (drug action at day 1 : drug action at day 4) × 100.

Statistical significance was calculated by using the paired *t*-test for 4 successive days. The significance level between the different diet groups (CA/CE, CA/DG, CA/DT, CA/DR, CA/DP, CE/DG, CE/DT, CE/DR, CE/DP) is $P < 0.01$.

* For details of diets, see Table 1.

The extent of the vitamin B depletion of rats was assessed from the activity of erythrocyte enzymes (Table 2), which are regarded as indicators of the nutritional status of the appropriate B-vitamin (Sauberlich, Skata & Dowdy, 1974).

There was a slight reduction in body-weight 4 d after administration of the drugs particularly in Zz. The weight losses were independent of the nutritional status of the rats with values in PB-treated rats of between 0 and 3 g and values of between 7 and 15 g in Zz rats, compared with no weight loss in the control group receiving saline injections.

Administration of drugs increased the weight of the rat livers. Again the effect was greater in Zz-treated rats (32–37 g liver/kg body-weight) than in PB-treated rats (28–38 g liver/kg body-weight). The value for control rats was 27–34 g liver/kg body-weight. The observed differences were not statistically significant. The differences in the dietary levels of the B-vitamins had no effect on liver weight.

From the in vivo results, it was obvious that the 'PB sleeping time' was increased ($P < 0.001$) by the vitamin B deficiencies, especially by the multiple deficiency (group DG) and that of riboflavin (group DR) (Table 3).

Hunger caused a prolonged 'PB sleeping time', as may be seen by comparison of the *ad lib.*-fed rats (group CA) with the hungry equal-fed rats (group CE) and vitamin B deficiencies enhanced the effect (group CE cf. groups DG, DR, DT, DP). In all groups repeated administration of PB led to an increased tolerance of the rats, expressed as 'per cent tolerance index' (PTI) in Table 3. In malnourished rats the increase in tolerance was higher than in control animals but it was not sufficient to reach the absolute values of the control animals.

Similar results were obtained for the 'Zz paralysis time' (Table 4), but with the difference that hunger (group CE) caused a shorter 'paralysis time' as is known for 'type-2' substrates.

The multiple vitamin B deficiency (group DG) increased the 'Zz paralysis time' significantly ($P < 0.001$) on each day and the same effect was observed for the riboflavin deficient group (DR). There was increased tolerance to repeated Zz injections, but less than to PB injections which was expected since Zz is a 'type-2' substrate. The PTI of vitamin B-deficient rats was not significantly altered.

The body temperature of the rats was independent of their nutritional status (34·0–36·0°)

Table 4. *'Paralysis time' after zoxazolamine injections (60 mg/kg intraperitoneally) on four successive days of rats given a control diet ad lib. (group CA) and equal-fed (group CE), and given diets deficient in several B-vitamins (group DG), and riboflavin (group DR)**

(Mean values and standard deviations)

Period after administration of drug (d) ...		'Paralysis time' (min)								
		1		2		3		4		
Group	*n*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	PTI
CA	8	65	7	55	4	49	2	47	1	138
CE	9	61	3	52	6	48	4	46	4	132
DG	10	75	7	65	7	60	4	55	4	136
DR	9	74	7	62	7	59	4	54	4	136

PTI, per cent tolerance index (drug action at day 1 : drug action at day 4) × 100.

Statistical significance was calculated by using the paired *t*-test for 4 successive days. The significance level between the different diet groups (CA/CE, CA/DG, CA/DR, CE/DG, CE/DR) is $P < 0.01$.

* For details of diets, see Table 1.

with a slight tendency to be lower in vitamin B-deficient rats. The reactions of the rat to the administration of drugs was evident in a significant hypothermia. The body temperature just at the beginning of the drug action was compared with the lowest value recorded, which was closest to the time of awakening. In PB-treated control rats this temperature difference was 1·3–1·6°, compared with 2·0–2·5° in vitamin B-deficient rats ($P < 0.025$). The difference between the four groups receiving the deficient diets were not significant. Zz reduced the body temperature more than did PB, but the effects of the vitamin B deficiencies were smaller. The Zz-treated control rats showed temperature decreases of 2·2–2·7° and those with vitamin B deficiencies were 2·5–3·0° ($P < 0.05$).

The concentrations of PB and Zz in the serum and brain of rats at awakening did not show differences when the values of the differently-fed groups were compared.

The protein contents of the liver and of the homogenates and the microsomal DME-containing supernatant fractions were the same for all groups of rats, therefore all results of the activities of the DME are expressed on a per g wet liver weight basis.

The activity of the UDP glucuronyltransferase was significantly ($P < 0.001$) lowered by vitamin B deficiency (Table 5); and riboflavin (group DR) had the greatest effect when compared with other B-vitamins tested. Hunger (group CE) stimulated the activity of this enzyme ($P < 0.001$). PB, but not Zz, caused an increased activity, which was not influenced by the nutritional variables. The activity of the hydroxylase of PB was decreased by all forms of vitamin B deficiencies tested. The effect was marked in multiple deficiency (group DG) and in riboflavin deficiency (group DR) ($P < 0.001$), and was less in thiamin deficiency (group DT) ($P < 0.05$) and in pyridoxine deficiency (group DP) ($P > 0.05$) (Table 5). Hunger (group CE) reduced its activity slightly ($P < 0.05$). PB induced the enzyme activity in all groups ($P < 0.001$); the effect was independent of the nutritional status of the rats. Even Zz had a similar though smaller inductive effect ($P < 0.01$).

The activity of the *N*-demethylase of aminopyrine was also decreased in multiple deficiency (group DG) and thiamin and riboflavin deficiency ($P < 0.05$), but not in pyridoxine deficiency (Table 5). Hunger also reduced its activity, but the observed differences did not appear statistically significant. PB ($P < 0.01$), but not Zz, had a stimulating effect on the enzyme activity. The extent of stimulation seemed to be higher in vitamin B-deficient groups than in the control groups.

Table 5. *Activities of drug-metabolizing enzymes in liver homogenates of rats given a control diet ad lib. (group CA) and equal-fed (group CE) and given diets deficient in several B-vitamins (group DG), thiamin (group DT), riboflavin (group DR), and pyridoxine (group DP)**

(The rats received on four successive days intraperitoneally injections of physiological saline (9 g NaCl/l) solution (5 ml/kg body-weight), pentobarbital (PB; 30 mg/kg) or zoxazolamine (Zz; 60 mg/kg). Mean values and standard deviations)

Group	Sub group	UDP glucuronyl-transferase† (*EC* 2.4.1.17)			PB hydroxylase‡			Aminopyrine-*N*-demethylase§			Zz hydroxylase‖			Aniline-hydroxylase¶ (*EC* 1.14.14.1)		
		Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
CA	NaCl	7·9	1·3	10	414	87	10	500	67	10	279	49	10	357	102	10
	PB	8·5	1·3	9	584	115	9	635	25	9	377	61	9	502	160	9
	Zz	7·9	1·8	8	510	115	8	497	61	8	310	69	8	431	201	8
CE	NaCl	9·7	2·2	9	378	90	9	403	88	12	383	87	9	441	61	12
	PB	11·0	2·9	10	535	101	10	526	104	12	480	78	10	696	116	12
	Zz	8·5	2·1	9	392	76	9	385	111	9	349	89	9	451	58	9
DG	NaCl	5·1	1·4	10	188	53	8	310	70	10	191	51	8	314	82	10
	PB	6·7	2·0	11	324	58	9	413	98	11	245	54	9	476	81	11
	Zz	4·9	1·5	10	276	67	10	276	65	10	213	56	10	329	57	10
DT	NaCl	5·7	1·6	11	326	110	11	333	69	11	248	57	11	460	89	11
	PB	6·8	1·6	13	450	138	13	499	129	13	334	62	13	725	116	13
DR	NaCl	5·0	1·4	10	238	54	11	349	95	11	200	31	11	355	27	11
	PB	6·9	1·5	11	401	105	12	464	91	12	249	35	12	518	87	12
	Zz	6·3	2·7	10	315	101	8	326	80	8	177	55	8	385	114	8
DP	NaCl	5·4	1·0	12	367	115	12	406	84	12	230	44	12	494	100	12
	PB	7·0	1·2	13	529	114	13	544	114	13	285	58	13	750	121	13

In calculating statistical significances either the two corresponding values (single subgroups) or data of the whole group (diet groups) were compared.

* For details of diets, see Table 1.

† μmol *p*-nitrophenol/min per g liver; '1470 *g* supernatant' fraction.

‡ nmol PB/30 min per g liver; '9000 *g* supernatant' fraction.

§ nmol $(formaldehyde)_2$/30 min per g liver; '9000 *g* supernatant' fraction.

‖ nmol Zz/30 min per g liver; '9000 *g* supernatant' fraction.

¶ nmol *p*-aminophenol/30 min per g liver; '9000 *g* supernatant' fraction.

The 'type-2' substrate enzymes (the hydroxylases of Zz and aniline) had their highest activity in hungry rats (group CE) ($P < 0.001$) (Table 5). The activity of both enzymes was lower in vitamin B-deficient groups, especially in multiple vitamin deficiency (group DG) and in riboflavin deficiency (group DR) ($P < 0.001$). Pyridoxine deficiency (group DP) had a more negative influence on the Zz hydroxylase ($P < 0.005$) compared with thiamin deficiency (group DT) ($P < 0.05$). The activity of aniline-hydroxylase was not significantly influenced by thiamin (group DT) and pyridoxine (group DP) when compared with the control group (CE). Again PB ($P < 0.01$), but not Zz, increased the enzyme levels, independently of vitamin availability.

The results of all in vivo measurements were correlated with the appropriate in vitro enzyme activity measurements. The results are shown in Table 6, which gives values for correlation coefficients for the several sets of values. For the PB values combined the correlation was comparatively high and significant, whereas that for Zz was low and not significant.

Table 6. *Correlation coefficient (r) for 'pentobarbital (PB) sleeping time' v activity of the hepatic PB hydroxylase, and for the 'zoxazolamine (Zz) paralysis time' v activity of the hepatic (Zz) hydroxylase, of rats given a control diet ad lib. (group CA) and equal-fed (group CE), and given diets deficient in several B-vitamins (group DG), thiamin (group DT), riboflavin (group DR), and pyridoxine (group DP)*†

	PB		Zz	
Group	*n*	*r*	*n*	*r*
CA	9	0·10NS	8	0·03NS
CE	34	0·23NS	18	0·12NS
DG	20	0·53*	20	0·22NS
DT	13	0·07NS		
DR	24	0·52*	8	0·34NS
DP	13	0·52*		
Total	113	0·59**	54	0·09NS

NS, not significant.
Statistical significance of *r*: *$P < 0.001$, ** $P < 0.05$.
† For details of diets, see Table 1.

DISCUSSION

One of the effects of vitamin B deficiency, especially of thiamin, is anorexia. This causes differences in energy intake of rats given *ad lib.* diets with different thiamin content. To study this effect of vitamin B deficiency would require an experimental system with multiple dietary variables. In order to overcome this problem, all rats received the same restricted amount of food, which was consumed completely within 24 h. The energy intake of rats in the different groups was therefore the same, and the changes in body-weight and organ weight were very similar for all groups. Even so, this feeding technique did not ensure that the vitamin intake was the only independent dietary variable. The rats of the vitamin B-deficient groups, especially groups DG and DT had little appetite and 'nibbled' their food, whereas the control rats (group CE) had no loss of appetite; they ate their food immediately and they were hungry the next morning at the next feeding. So two different control groups (*ad lib.*-fed (CA) and equal-fed (CE)) were used in this study, which made it possible to differentiate between the effects of hunger and of the B-vitamins on drug metabolism.

The metabolism of 'type-1' substrates was reduced by energy deficiency (group CE *v* group CA): the 'PB sleeping time' was increased, the activities of the PB hydroxylase and of the aminopyrine-*N*-demethylase were decreased. The opposite was observed with 'type-2' substrates; the 'Zz paralysis time' decreased and the activities of the hydroxylases of Zz and aniline increased. These results are in full agreement with those of Kato & Tanaka (1967). The observation that in energy-deficient animals (group CE) the activity of the UDP glucuronyltransferase is increased was also reported by Mietinnen & Leskinen (1963). The 'equal-feeding' technique ensured that the protein content of the rat livers was the same in all groups. Differences were observed only in comparison with *ad lib.*-feeding (Grosse & Wade, 1971), which suggests they are associated with the different energy intake or total food consumption, respectively, since with comparable food consumption, differences only in the intakes of thiamin (Wade, Wu & Lee, 1975) or riboflavin (Patel & Pawar, 1974) did not change the protein levels of the livers.

The observed alterations of the drug metabolism due to the vitamin B deficiencies were significant despite the hunger effect previously mentioned. The 'sleeping time' after PB administration was increased most with multiple deficiency (group DG) and with riboflavin deficiency (group DR). Deficiency of thiamin (group DT) or of pyridoxine (group DP)

influenced the 'sleeping time' only to a minor extent. Very similar results have been reported for the action of thiopental which is increased in multiple vitamin B deficiency (Higgins & Mann, 1943) and in riboflavin and pyridoxine deficiency, but less in thiamin deficiency (Levy & Di Palma, 1953, Catz *et al.* 1970). The 'hexobarbital sleeping time' is little affected by a thiamin deficiency (Grosse & Wade, 1971).

The action of the 'type-2' substrate Zz was also increased by B-vitamin deficiencies, and again the main effect was seen in groups DG and DR. The results of studies on thiamin-deficient rats (Grosse & Wade, 1971) are in contrast to these reported here in that a shorter 'Zz paralysis time' was found. It may be that the hunger effect was greater than it was in the present study.

The increasing tolerance that resulted from repeated drug applications was independent of the vitamin B status of the rats. The effect was slightly greater in malnourished rats, but it was never enough to balance the deficit completely when comparing the absolute values of drug action in control and vitamin B-deficient rats. The application of drugs caused hypothermia in the rats, more so with Zz than with PB. The effects of the drugs were enhanced in vitamin B deficiencies. The in vivo results showed that B-vitamin deficiencies made the rat more susceptible to both drugs. The main effect of a multiple deficiency was due to the lack of riboflavin; pyridoxine deficiency had a smaller effect, and thiamin deficiency had very little influence.

The increased reactions of drugs in vitamin B deficiency could be caused by altered reactions between the drugs and their receptors, e.g. those in the central nervous system. If so, then the concentrations of the drugs in serum or brain at awakening of the rats should be different. The results of these determinations showed no significantly different drug levels in serum and brain for the different groups. So the differences in drug reaction are better explained by a lowered metabolism of the drugs in vitamin B deficiencies. The in vitro measurements showed clearly that the activities of the tested hepatic DME were reduced. The activity of the *N*-demethylase of aminopyrine was less reduced than that of the hydroxylases. There was no difference between the B-vitamins in their effects on the enzymes of 'type-1' and 'type-2' substrates.

The results of repeated drug administration on the activities of DME revealed the expected difference between 'type-1' substrate PB and 'type-2' substrate Zz. PB caused a significant increase in the activities of the tested DME regardless of vitamin B availability. The rate of induction was greater with the 'type-1' substrate enzymes (PB-hydroxylase and aminopyrine-*N*-demethylase) than with the 'type-2' substrate enzymes, but even in these the induction effect of PB was obvious. Unlike PB, the 'type-2' substrate Zz did not show inductive potency. This is in accordance with investigations of Conney (1967) and Schmid (1968), but it is in contrast with the in vivo finding that the rats became more tolerant to Zz after repeated applications.

Comparing the effects of the three individual B-vitamin deficiencies on the activities of the DME with those of the multiple deficiency, it is obvious from the in vivo results that riboflavin deficiency was mainly responsible for the losses in enzyme activities. The results for group DR were rather similar to those for group DG. Only with UDP glucuronyltransferase did it seem that all the three B-vitamins had a similar effect. As mentioned previously the *N*-demethylase of aminopyrine was less influenced by vitamin B deficiencies. This is in accordance with other investigations which showed that riboflavin deficiency has smaller effects on *N*-demethylases compared with its effect on the hydroxylases of benzpyrene and on azo- and nitroreductases (Catz *et al.* 1970; Williams, Grantham, Yamamoto & Weisburger, 1970; Shargel & Mazel, 1973). Riboflavin deficiency reduced the activities of all three hydroxylases tested. These results are similar to those of Patel & Pawar (1973, 1974), who worked with rats, but disagree with those of Catz *et al.* (1970), who worked with mice.

The disparity is possibly due to the different animal species used, but also due to different feeding practices, since the mice but not the rats were fed *ad lib*. The influence of thiamin and pyridoxine on DME was smaller than that of riboflavin. Pyridoxine deficiency had some effect in lowering the activities of the enzymes of 'type-2' substrates tested and of the glucuronidation, but no effect of those of 'type-1' substrates. Thiamin deficiency had almost the opposite effect, the enzymes of 'type-1' substrates were influenced negatively, and those of 'type-2' substrates to a different degree.

The in vitro enzyme-activity tests showed that the activities of DME were reduced by vitamin B deficiencies, but they cannot explain the mechanisms of the observed changes. It is not possible to decide whether the enzyme activities were decreased because of a smaller amount of the enzymes (changes in anabolism, or catabolism, or both) or because of alterations in their specific activity (e.g. by altered availability of co-factors or changes of the apoenzyme configuration) or both these factors. It is probably significant that the microsomal enzyme complex contains the flavoproteins NADPH-cytochrome *c*-reductase (*EC* 1.6.2.4) and NADH-cytochrome *b*-reductase (*EC* 1.6.2.2), both of which seem to be influenced by riboflavin deficiency (Shargel & Mazel, 1973; Patel & Pawar, 1974).

The correlations between the in vivo results ('sleeping time') and the in vitro results (activity of the hydroxylase of PB) were comparatively high, and seemed to be independent of nutritional status. The results of in vivo determinations, which are comparatively quick and cheap, can be taken as indication of the activities of the appropriate hepatic mixed-function oxidases of 'type-1' substrates. In contrast to 'type-2' substrates, in vivo and in vitro results were almost unrelated. There was the anomaly that whereas in vitamin B deficiencies the drug action was increased, and repeated Zz applications caused increasing tolerance, the enzyme activity in the liver did not reflect these changes. The concentrations of Zz in serum and in brain of the rats at awakening did not indicate an altered interaction of Zz with the receptors. The explanations of the observed, apparently contrasting, results of Zz probably have another, less straightforward or multicausal mechanism as they were possible for 'type-1' substrates. None of the in vivo determinations proved a useful indicator of the activity of hepatic enzymes with 'type-2' substrates.

Finally it can be concluded that deficiencies of B-vitamins caused an increased action of drugs, such as PB and Zz. For 'type-1' substrate the effect could be explained by the reduced drug metabolism in the liver. For 'type-2' substrates the observed over-all effects on rats could not be explained by an altered activity of the DME. The key vitamin in all the observed changes was riboflavin, a deficiency of which caused a loss of activity in all the mixed-function oxidases and in the UDP glucuronyltransferase.

In vitamin B deficiency syndromes, and especially in riboflavin deficiency, it is advisable to monitor drug application carefully. The safety evaluation of foreign chemicals should not neglect the nutritional variables, and should not be tested exclusively with well-fed animals, but also with malnourished animals, which could be more sensitive.

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