https://doi.org/10.1079/BJN19890149 Published online by Cambridge University Press

Effects of vitamin B_6 deficiency and repletion on the uptake of steroid hormones into uterus slices and isolated liver cells of rats

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(*Received 15 August 1988 – Accepted 5 December 1988*)

1. In vitro, pyridoxal phosphate extracts steroid-hormone receptors from tight nuclear binding (Cidlowski & Thanassi, 1981); in vitamin B_6-deficient rats there is increased and prolonged nuclear accumulation of oestradiol in the uterus and testosterone in the prostate, associated with enhanced biological responsiveness of these target tissues to steroid hormone action (Symes *et al.* 1984; Bowden *et al.* 1986).

2. Slices of uterus from vitamin B_6-deficient rats accumulated more [^{3}H]oestradiol than did tissue from repleted animals. Acute repletion with vitamin B_6 (0·5–1 h before killing) further increased the uptake of the steroid.

3. Isolated hepatocytes from vitamin B_6 - deficient rats accumulated more [^{3}H]dexamethasone than did cells from repleted animals. Pre-incubation of the hepatocytes with pyridoxal phosphate resulted in a further increase in the uptake of the steroid.

4. The results suggest that in addition to the putative role of pyridoxal phosphate in releasing steroid-hormone–receptor complexes from tight nuclear binding (Cidlowski & Thanassi, 1981), vitamin B_6 deficiency may also increase the concentration of steroid-hormone receptors or enzymes and other steroid-binding proteins in target tissues.

In addition to its well-established role as a co-enzyme for glycogen phosphorylase (*EC* 2.4.1.1) and many enzymes involved in amino acid metabolism, the metabolically active vitamer of vitamin B_6, pyridoxal phosphate, appears to have a role in the action of steroid hormones.

Steroid hormones act by inducing the synthesis of specific enzymes and other proteins in target tissues. The steroid enters the nucleus and induces transcription of DNA to mRNA, which is then translated on the ribosomes, yielding the new proteins. Target-tissue specificity is ensured by the presence of hormone-receptor proteins which are responsible for both the nuclear uptake of the steroid and the interaction with DNA and nucleoproteins.

A number of studies have shown that pyridoxal phosphate will extract steroid-receptor proteins from the nuclei of target tissues. This has been demonstrated not only for the oestrogen receptor (Muller *et al.* 1980; Seeley *et al.* 1984) but also for the androgen- (Isomaa *et al.* 1982), progesterone- (Chen *et al.* 1981) and glucocorticoid- (Cidlowski & Thanassi, 1978, 1981) receptor proteins. Reaction with pyridoxal phosphate also inhibits the binding of steroid–receptor complexes to isolated DNA and chromatin. Again the effect has been demonstrated for oestrogen (Muldoon & Cidlowski, 1980; Muller *et al.* 1980), androgen (Hiipakka & Rao, 1980; Mulder *et al.* 1980), progesterone (Nishigori *et al.* 1978) and glucocorticoid (Cake *et al.* 1978; Majumder *et al.* 1983) receptors.

These effects are specific for the phosphorylated vitamer, suggesting that there may be a specific pyridoxal phosphate binding site on the receptor proteins. Furthermore, they are apparent at low concentrations of pyridoxal phosphate, of the same order of magnitude as occur in tissues under normal conditions. This specificity of the effects of pyridoxal phosphate, but not pyridoxal, and the low concentrations at which they can be observed, led Cidlowski & Thanassi (1981) to propose that pyridoxal phosphate may have a

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physiological role in the action of steroid hormones. They suggested that it might act as a cofactor in the release of hormone–receptor complexes from tight nuclear binding, thus terminating hormone action, releasing the steroid from the nucleus, and freeing or recycling receptors for further uptake of steroid if hormone stimulation continues.

Previous studies in this laboratory (Holley *et al.* 1983; Symes *et al.* 1984; Bowden *et al.* 1986) have demonstrated that this in vitro interaction between vitamin B_6 and steroid-hormone receptors may indeed be physiologically and nutritionally relevant. Vitamin B_6 deficiency in the rat results in an increase in the uptake and relative nuclear accumulation of [^{3}H]oestradiol in such target tissues as the uterus, liver and hypothalamus (Holley *et al.* 1983). Vitamin B_6 deficiency also results in enhanced sensitivity of target tissues to stimulation by submaximal doses of oestrogens: increased uterus growth and induction of uterine peroxidase, and much increased suppression of luteinizing hormone compared with vitamin B_6-supplemented control animals (Bowden *et al.* 1986). In vitamin B_6-deficient male rats there is a similar increase in the nuclear accumulation of [^{3}H]testosterone in the prostate, and again this is accompanied by increased sensitivity of target tissues to androgen action, with a marked increase in the prostate mitotic index in response to low doses of testosterone (Symes *et al.* 1984; Bender *et al.* 1987).

The present study was undertaken in order to determine whether the increased target tissue uptake of, and responsiveness to, steroid hormones in vitamin B_6 deficiency is entirely a result of low tissue concentrations of pyridoxal phosphate, in which case acute repletion with vitamin B_6 would be expected to normalize hormone uptake, or whether there might be more long-term responses of steroid-hormone receptors to vitamin B_6 deficiency. The uptake of [^{3}H]oestradiol into slices of uterus, and of the synthetic glucocorticoid analogue [^{3}H]dexamethasone into isolated hepatocytes has been studied in tissues from vitamin B_6-deficient and repleted rats.

MATERIALS AND METHODS

Animals and diets

Wistar rats bred in University College London were used in the present study. Ovariectomized females, maintained on a replacement dose of oestradiol, were used for the studies of uptake of oestradiol into uterus slices, and males were used for the study of glucocorticoid uptake into isolated hepatocytes. They were weaned at 21 d after birth onto the vitamin B_6-deficient high-protein diet described previously by Symes *et al.* (1984). Previous studies have demonstrated that after 4 weeks the animals show a significant degree of vitamin B_6 depletion, with a decrease in the liver content of pyridoxal phosphate from 15·4 (SE 0·25) to 6·5 (SE 0·15) nmol/g (Symes *et al.* 1984; Bowden *et al.* 1986). Repleted animals received the same diet, but for 3 d before the day on which they were killed they received 1 mg pyridoxine hydrochloride/kg body-weight by oral intubation daily at 09.00 hours. Previous studies (Bowden, 1985; D. A. Bender, unpublished results) have shown that repletion with vitamin B_6 for 1–3 d restores the uptake of [^{3}H]oestradiol into the uterus or of [^{3}H]testosterone into the prostate (both of which are increased in deficient animals) to the same level as in animals maintained from weaning on a vitamin B_6-supplemented diet.

Animals which were acutely repleted with vitamin B_6 received the same dose of pyridoxine hydrochloride (1 mg/kg body-weight) by oral intubation at intervals between 0·5 and 5 h before they were killed. During this time they were not allowed access to food.

After they had received the vitamin B_6-free diet for 3 weeks, female rats were ovariectomized by the dorsal route under anaesthesia with Hypnorm (Janssen; fluanisone 10 mg and fentanyl citrate 0·135 mg/kg body-weight, intramuscularly) and diazepam (2·5

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mg/kg body-weight, intraperitoneally). From the third day post-operatively, all animals received a subcutaneous injection of 3 μg oestradiol/kg body-weight, dissolved in maize oil, daily at 09.00 hours for 5 d, in order to maintain oestrogen receptors and oestrogen-sensitive tissue in the uterus. They were killed 24 h after receiving the last dose of oestradiol.

In the animals used for studies of uterine uptake of [^{3}H]oestradiol, portions of liver, kidney and skeletal muscle were dissected out when the animals were killed, and were frozen and stored at −20° for up to 1 week before being used for determination of pyridoxal phosphate by the modification of the cyanide fluorimetric method (Adams, 1979) described previously by Bender *et al.* (1982).

[^{3}H]oestradiol uptake into uterus slices

Animals were killed by cervical dislocation, and the uterus was dissected out and cooled in ice-cold Krebs–Ringer phosphate–bicarbonate buffer. It was then trimmed of fat, and cut into small rings, each weighing approximately 5 mg, using a tissue chopper made from a stack of razor blades separated by 1 mm thick washers. These slices were then washed in ice-cold Krebs–Ringer phosphate–bicarbonate buffer. Four slices from each uterus were frozen and stored at −20° for determination of pyridoxal phosphate as described previously.

For incubation, four slices were incubated at 37° for periods of 1–8 min in Krebs–Ringer phosphate–bicarbonate buffer containing 5 mmol glucose/l and 9 pmol [6,7-^{3}H]oestradiol/ml (diluted to a specific activity of 94 Ci/mol with non-radioactive oestradiol; Amersham International plc, Amersham, Bucks), under an atmosphere of oxygen–carbon dioxide (95:5 v/v). At the end of the incubation period, the tissue slices were separated on a coarse nylon mesh, and were washed with ice-cold Krebs–Ringer phosphate–bicarbonate buffer, blotted and washed again with ice-cold Krebs–Ringer phosphate–bicarbonate buffer, in order to remove as much as possible of the radioactive incubation medium. They were then suspended in 1 ml ice-cold Tris–sucrose buffer (0·25 mol sucrose and 10 mmol Tris hydrochloride/l at pH 7·4, containing 1·5 mmol EDTA and 2 mmol 2-mercapto-ethanol/l) and homogenized using a Polytron tissue emulsifier at low speed for 30 s.

Duplicate 0·1 ml portions of the homogenate were frozen for determination of DNA by the diphenylamine–acetaldehyde colorimetric method of Burton (1956), and the remainder was centrifuged at 2000 ***g*** for 20 min at 4°. Duplicate 0·2 ml portions of the supernatant fraction were mixed with 2 ml of Scintran Cocktail-T water-miscible scintillation cocktail (BDH, Poole, Dorset) for determination of radioactivity by liquid-scintillation counting. The crude nuclear pellet was washed twice by resuspending in 1 ml ice-cold Tris–sucrose buffer, and centrifuged at 2000 ***g*** for 20 min. The final pellet was then dissolved in 0·5 ml Scintran tissue solubilizer (BDH, Poole, Dorset) and mixed with 2 ml of a solution of 5 g PPO and 0·5 g POPOP/l toluene for determination of radioactivity by liquid-scintillation counting.

The results were expressed as radioactivity in the supernatant fraction and crude nuclear fractions/mg DNA in the complete incubation.

[^{3}H]dexamethasone uptake into isolated hepatocytes

In order to avoid circadian variations in glucocorticoids and receptors, all animals were killed between 08.30 and 09.00 hours. They were anaesthetized with 150 mg pentobarbitone/kg body-weight, and hepatocytes were prepared by portal–caval perfusion of the liver with collagenase (*EC* 3.4.24.3) as described previously (Bender & Olufunwa, 1988), a modification of the methods described by Elliot *et al.* (1976) and Romero & Viña (1983), except that once recirculation of the perfusion medium was commenced, it was brought to 2·5 mmol calcium ions/l. This modification permits the preparation of a good yield of

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metabolically active hepatocytes in a shorter time-period, and using a lower concentration of collagenase, than the procedure used previously, in which the perfusion medium was Ca-free throughout.

After washing in ice-cold Krebs–Ringer phosphate–bicarbonate buffer, the hepatocytes were suspended in ice-cold Krebs–Ringer phosphate–bicarbonate buffer containing 5 mmol glucose, 4·5 mmol lactate and 0·5 mmol pyruvate/l (with or without 500 nmol pyridoxal phosphate/l), to give 10–20 mg dry weight of cells/ml. They were incubated for 20 min at 37°, then cooled on ice until required. All incubations were performed in glassware which had been siliconized with a solution of dimethyl dichlorosilane in carbon tetrachloride (20 ml/l) in order to prevent cells adhering to the glass.

Portions (0·5 ml) of the cell suspension were then pre-incubated at 37° for 10 min, when 0·5 ml of a solution of 200 nmol [1,2,4,6,7-^{3}H]dexamethasone/l (diluted to a specific activity of 9·4 Ci/mol with non-radioactive dexamethasone; Amersham International plc) in Krebs–Ringer phosphate–bicarbonate buffer was added, and the incubation allowed to continue for periods of 1–16 min.

At the end of the incubation, the incubation mixture was poured into a 1·5 ml Eppendorf centrifuge tube, over 0·5 ml of silicone oil (2 vol. silicone fluid DC550:1 vol. dinonyl phthalate), and centrifuged at 12000 ***g*** for 1 min. After decanting off the supernatant fraction, and wiping the sides of the centrifuge tube, the cell pellet was suspended in 2 ml Scintran Cocktail-T water-miscible scintillation fluid for measurement of radioactivity in whole cells.

For determination of nuclear uptake of [^{3}H]dexamethasone, the cell pellet was resuspended in 1 ml of a solution of 1 g Nonidet P-40 non-ionic detergent/l (BDH) in Hepes (10 mmol/l), adjusted to pH 7·9 with 1 mol sodium hydroxide/l, containing 10 mmol sodium chloride/l and 3 mmol magnesium chloride/l, to lyse the cells. The cells were then centrifuged again at 12000 ***g*** for 1 min, and the resultant nuclear pellet was then suspended in Cocktail-T scintillation fluid, as described previously. Preliminary studies showed that this procedure gave more or less complete lysis of the cells, with little or no damage to the nuclei, as assessed by light microscopy.

The results were expressed as radioactivity in whole cells or isolated nuclei/mg dry weight of cells in the original incubation.

Statistical methods

Incubations of slices of uterus or isolated hepatocytes were performed in duplicate on preparations from any one animal at each time-point. The means of these duplicates were then used for calculation of the means from eight animals in each group for studies with uterus slices, and five animals in each group for studies with isolated hepatocytes. The curves shown in Figs. 2 and 3 were drawn by inspection.

RESULTS

Fig. 1 shows that maintenance on the high-protein vitamin B_6-free diet for 4 weeks resulted in a significant depletion of pyridoxal phosphate in liver, kidney, uterus and skeletal muscle. Following oral administration of 1 mg pyridoxine hydrochloride/kg body-weight the concentration of pyridoxal phosphate in the liver rose above the control level, and that in the uterus to the control level, within 1 h, while kidney pyridoxal phosphate did not return to the control level until between 2 and 5 h after the oral dose, and muscle pyridoxal phosphate showed little or no short-term response. The concentration of pyridoxal phosphate in the livers of animals repleted with pyridoxine hydrochloride for 3 d before

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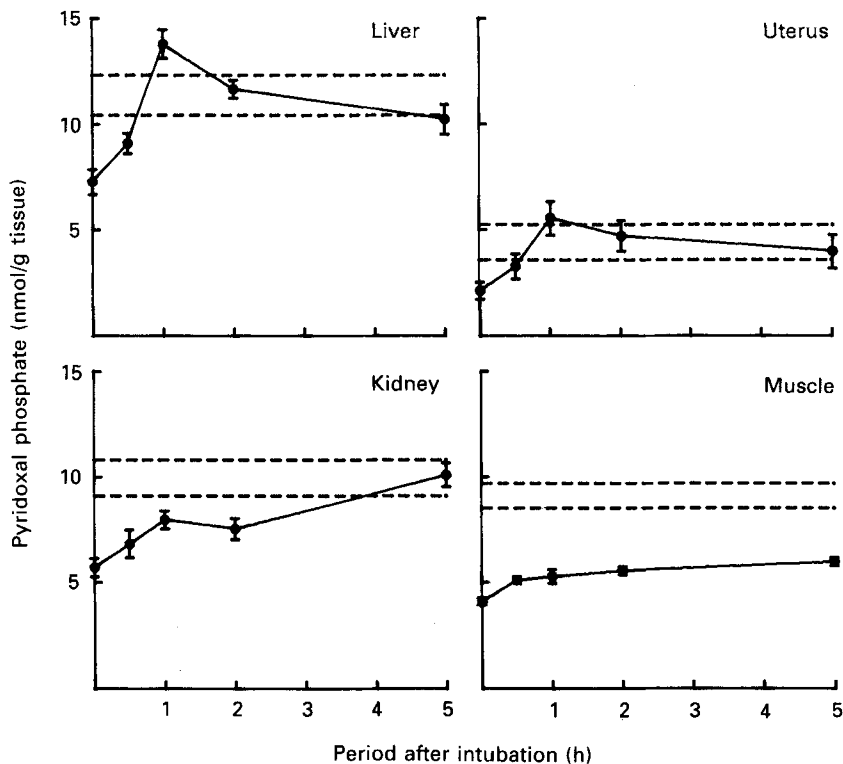

Fig. 1. Tissue concentrations of pyridoxal phosphate in vitamin B_6-deficient female rats and animals repleted with 1 mg pyridoxine hydrochloride/kg body-weight by oral intubation at varying times before killing. Points are means, with their standard errors represented by vertical bars, for duplicate determinations on tissue samples from eight animals in each group; (----), means with their standard errors for control animals repleted with vitamin B_6 3 d before killing. For details of procedures, see p. 621.

killing was the same as that reported previously in animals maintained for 4 weeks from weaning on the diet as used in the present study, but supplemented with 5 mg pyridoxine hydrochloride/kg diet (Bowden *et al.* 1986)

The uptake of [^{3}H]oestradiol into slices of uterus is shown in Fig. 2. Tissue from vitamin B_6-deficient rats showed a higher uptake of [^{3}H]oestradiol into the supernatant fraction than that from 3 d repleted animals, throughout the 8 min incubation period. Acute repletion of deficient animals with vitamin B_6 30 min before killing resulted in a further increase in the uptake of [^{3}H]oestradiol into the supernatant fraction. Uptake of [^{3}H]oestradiol into the crude nuclear fraction was unaffected by vitamin B_6 deficiency or repletion for the first 4 min of incubation; thereafter, tissue from deficient animals showed greater uptake than that from 3 d repleted animals. Again acute repletion with vitamin B_6 resulted in a further increase in uptake. Nuclear uptake of [^{3}H]oestradiol was maximal after 4 min in control animals, while in tissue from vitamin B_6-deficient animals the uptake was maximal at 6 min, and in tissue from acutely repleted animals the nuclear uptake of [^{3}H]oestradiol continued to increase until 7 min.

In tissue from deficient animals acutely repleted with vitamin B_6 for longer periods (values not shown), uptake of [^{3}H]oestradiol into both the supernatant and crude nuclear

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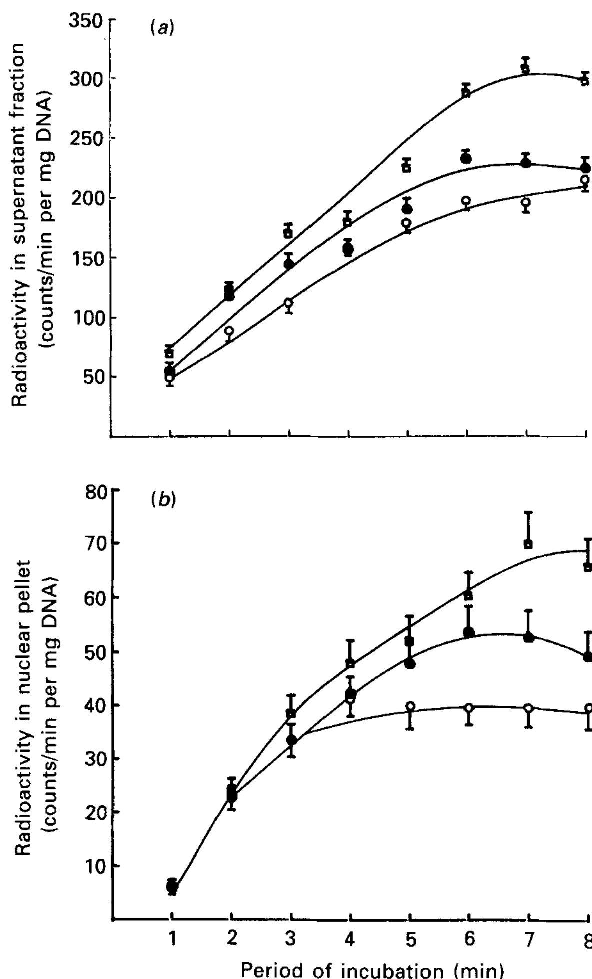

Fig. 2. Uptake of [^{3}H]oestradiol (counts/min per mg DNA in original tissue homogenate) into slices of rat uterus. (○–○), Tissue from control animals, repleted with vitamin B_6 3 d before killing; (●–●), tissue from vitamin B_6-deficient animals; (□–□), tissue from deficient animals repleted with vitamin B_6 30 min before killing. (*a*) Uptake into the supernatant fraction and (*b*) uptake into the crude nuclear fraction. Points are means, with their standard errors represented by vertical bars, for duplicate determinations on tissue samples from eight animals in each group. For details of procedures, see p. 621.

fractions was the same at 60 min after repletion as that at 30 min. Thereafter it declined so that 5 h after repletion it was lower than that in deficient animals, although slightly higher than that in tissue from the 3 d repleted (control) animals.

As shown in Fig. 3, vitamin B_6 deficiency had a more marked effect on the uptake of [^{3}H]dexamethasone into isolated hepatocytes. Cells from deficient animals accumulated about twofold more [^{3}H]dexamethasone than did cells from 3 d repleted animals, with a threefold increase in nuclear uptake. Pre-incubation of cells from either vitamin B_6-deficient or 3 d repleted animals resulted in an increase in the uptake of [^{3}H]dexamethasone into whole cells; uptake into the nuclear fraction was only enhanced by pre-incubation with pyridoxal phosphate in cells from deficient animals, with a slight enhancement in early nuclear uptake in cells from repleted animals.

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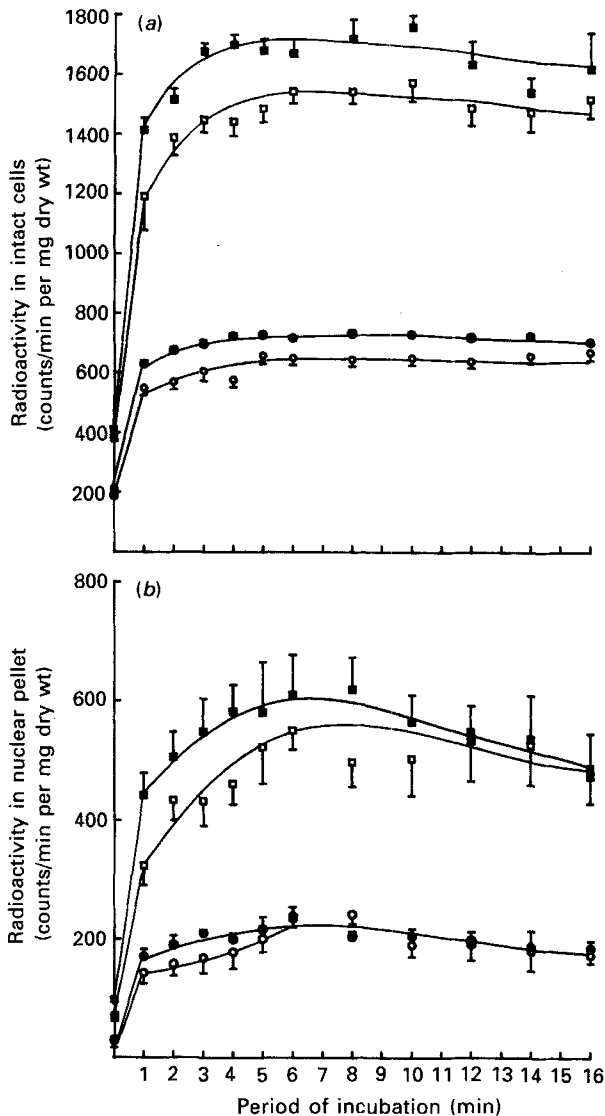

Fig. 3. Uptake of [^{3}H]dexamethasone (counts/min per mg dry weight of cells in original incubation) into isolated rat hepatocytes. (○–○), Hepatocytes from control animals repleted with vitamin B_6 3 d before killing; (●–●), hepatocytes from control animals pre-incubated for 20 min with 500 nmol pyridoxal phosphate/l; (□–□), hepatocytes from vitamin B_6-deficient animals; (■–■), hepatocytes from vitamin B_6-deficient animals pre-incubated for 20 min with 500 nmol pyridoxal phosphate/l. (*a*) Uptake into intact cells and (*b*) uptake into the isolated nuclei. Points are means, with their standard errors represented by vertical bars, for duplicate determinations on tissue samples from five animals in each group. For details of procedures see p. 621.

DISCUSSION

After oral administration of 1 mg pyridoxine hydrochloride/kg body-weight, it is likely that most will be taken up by the liver; in any case, extrahepatic tissues do not normally take up pyridoxine from the circulation. Rather, they are reliant on pyridoxal phosphate released by the liver, which is dephosphorylated to pyridoxal extracellularly; pyridoxal enters tissues by passive diffusion, and is then trapped by phosphorylation to pyridoxal

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phosphate (Lumeng *et al.* 1974). Both uterus and kidney showed some increase in pyridoxal phosphate 30 min after repletion, suggesting that there was significant export of pyridoxal phosphate from the liver even before the liver content had been restored to the control level.

The slow response of muscle pyridoxal phosphate is to be expected. Muscle contains about 80% of the total body vitamin B_6, and most of this is associated with glycogen phosphorylase (Black *et al.* 1975). It is not released from muscle in vitamin B_6 deficiency, although it is released, and redistributed among other tissues, during food deprivation as muscle glycogen reserves decrease, so reducing the requirement for pyridoxal phosphate for muscle glycogen metabolism and increasing the requirement for transamination of gluconeogenic amino acids in other tissues. This is the result of catabolism of glycogen phosphorylase (Black *et al.* 1978). The lower muscle content of pyridoxal phosphate in the deficient animals is thus presumably a response to their low food intake; in previous studies we have shown that animals fed on the vitamin B_6-deficient diet eat less total food, and grow more slowly, than those fed on a vitamin B_6-supplemented diet (Bowden *et al.* 1986). Hence, short-term repletion of deficient animals with vitamin B_6 would not be expected to increase the muscle content of pyridoxal phosphate; this will only occur as new glycogen phosphorylase protein is synthesized. By contrast, many enzymes of amino acid metabolism, which contain the major proportion of pyridoxal phosphate in other tissues, remain as catalytically inactive apo-enzymes during vitamin B_6 deficiency, and indeed some enzymes respond to vitamin B_6 deficiency by greatly increased synthesis of the apo-enzyme (Bayoumi *et al.* 1972). These apo-enzymes will be able to sequester a relatively large amount of pyridoxal phosphate more or less immediately after acute repletion.

The design of the present study, where all animals were maintained on the vitamin B_6-free diet for 3–4 weeks, then controls were repleted with the vitamin for 3 d, was intended to eliminate the long-term effects of impaired growth, and possibly delayed development on hormone uptake. However, it is not possible to exclude short-term (1–3 d) effects of vitamin repletion on food intake, and hence on protein synthesis or the synthesis, target tissue uptake or actions of steroid hormones, following repletion. In the studies with slices of uterus tissue, where animals were repleted with vitamin B_6 0·5–5 h before killing, they were deprived of food after repletion, so changes in food intake cannot be a factor in the interpretation of these results. In the studies with isolated hepatocytes, repletion with vitamin B_6 was performed in isolated cells, which were incubated for only a short period, without the addition of any substrates to permit significant protein synthesis.

The increased uptake of [^{3}H]oestradiol into slices of uterus in tissue from vitamin B_6-deficient animals (Fig. 2) is qualitatively the same as seen in previous studies with intact animals (Holley *et al.* 1983; Bowden *et al.* 1986), although much less marked. There is relatively little oestrogen-sensitive tissue compared with the larger amounts of muscle and connective tissue, in the rat uterus. The results may thus reflect interference by non-specific binding of the steroid, as well as poor access of the [^{3}H]oestradiol in the incubation medium to the oestrogen-sensitive endometrial cells in the lumen of the tissue slices.

In isolated hepatocytes (Fig. 3), where access to cells is unimpeded in the incubation medium, and there is little tissue damage, vitamin B_6 deficiency had a very much more marked effect on the uptake of [^{3}H]dexamethasone. Indeed, the twofold increase in steroid accumulation in hepatocytes from deficient animals was greater than has been observed for [^{3}H]oestradiol uptake into the uterus, or [^{3}H]testosterone uptake into the prostate in studies in intact animals (Symes *et al.* 1984; Bowden *et al.* 1986).

The effects of vitamin B_6 deficiency are in agreement with the suggestion that pyridoxal phosphate may function in vivo to release steroid–receptor complexes from tight nuclear binding, so removing the steroid from the nucleus, and terminating its actions (Cidlowski

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& Thanassi, 1981). Hence, in vitamin B_6 deficiency the steroid would be expected to remain tightly bound in the nucleus for longer, thus increasing and prolonging the nuclear uptake, and increasing biological responses, as has been observed in vitamin B_6-deficient animals (Symes *et al.* 1984; Bowden *et al.* 1986). However, it is difficult to account for the increase in extranuclear accumulation of steroid in vitamin B_6 deficiency by such a mechanism.

Similarly, extraction of receptors from tight nuclear binding by pyridoxal phosphate cannot readily account for the observed increase in both total and nuclear accumulation of radioactive steroid in response to short-term repletion of deficient animals (0·5–1 h) or pre-incubation of isolated hepatocytes with pyridoxal phosphate. It is possible that steroids enter target cells by way of a specific permease; however, there is little evidence for this suggestion, and as lipophilic molecules they would enter readily by diffusion. It must be assumed that within the cell, and certainly within the nuclear compartment, they will always be associated with receptors or other proteins.

It, therefore, seems likely that in addition to extraction of the steroid–receptor complex from tight nuclear binding (Cidlowski & Thanassi, 1981), which is assumed to be impaired in vitamin B_6 deficiency due to low intracellular concentrations of pyridoxal phosphate, there is a longer-term effect of vitamin B_6 deficiency on steroid-hormone-binding proteins. From the present results, which show an increased total cell capacity for steroid uptake in vitamin B_6 deficiency, it seems likely that there is an increase in the total cell content of such proteins. Following acute repletion of deficient animals with pyridoxal phosphate, which will release receptors from tight nuclear binding, freeing them to take up the labelled steroid, there seems to be a gradual decrease in the content of receptors over 2–5 h. This suggests that pyridoxal phosphate may have an effect on the synthesis or catabolism of steroid-hormone-binding proteins. From the present results it is not possible to determine whether this is an effect on the high-affinity steroid-binding proteins (steroid-hormone receptors) or on the low-affinity binding proteins (other proteins, and especially enzymes which metabolize steroids, so-called 'non-specific' binding).

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Printed in Great Britain

https://doi.org/10.1079/BJN19890149 Published online by Cambridge University Press