Biochem. J. (1973) **134**, 817–820
Printed in Great Britain

Regulation of Vitamin D Metabolism: Factors Influencing the Rate of Formation of 1,25-Dihydroxycholecalciferol by Kidney Homogenates

By KAY W. COLSTON, IMOGEN M. A. EVANS, LEE GALANTE, IAIN MACINTYRE and D. W. MOSS
Endocrine Unit, Department of Chemical Pathology, Royal Postgraduate Medical School, London W12 0HS, U.K.

(*Received* 19 *April* 1973)

The formation of 1,25-dihydroxycholecalciferol from 25-hydroxycholecalciferol by chick kidney homogenates is inhibited by increasing concentrations of Ca^{2+}. The apparent K_m for the hydroxylation reaction is 1×10^{-7}M, significantly lower than that reported for isolated mitochondria. Separated cytoplasmic and particulate fractions are inactive, but on recombination activity is restored, possibly because of the presence in the soluble fraction of a factor with a high affinity for 25-hydroxycholecalciferol.

Fraser & Kodicek (1970) demonstrated an enzyme system in vertebrate kidney that converts 25-hydroxycholecalciferol into its most active metabolite, 1,25-dihydroxycholecalciferol. Both whole-tissue homogenates and a particulate fraction (characterized as 'large' mitochondria) have been shown to carry out the reaction (Fraser & Kodicek, 1970; Gray *et al.*, 1972), which is inhibited by Ca^{2+} (Galante *et al.*, 1972*b*; Fraser & Kodicek, 1973).

We have now extended our preliminary studies of inhibition by Ca^{2+}, and have also shown that a cytoplasmic factor or factors markedly enhances the reaction in homogenates of chick kidney. One of these factors may be identical with a substance, located in the cytoplasm of this tissue, that binds 25-hydroxycholecalciferol. Its presence may account for some of the differences that have been observed between particulate preparations and whole-tissue homogenates in the substrate concentrations required to support the reaction.

Experimental

Preparation of kidney homogenates. One-day-old white Leghorn chicks were fed on a vitamin D-deficient diet (calcium 0.34%, phosphorus 0.54%) for 3–4 weeks. The chicks were killed by decapitation and the kidney tissue was removed immediately and placed on ice. Homogenates (2%, w/v) were prepared, in a Potter–Elvehjem homogenizer, in ice-cold 15mM-Tris – acetate buffer, pH7.4, supplemented with $MgCl_2$ (1.9mM), sodium succinate (5mM) and sucrose (200mM).

Assay of 25-hydroxycholecalciferol 1-hydroxylase activity. Portions (3ml) of homogenates were preincubated at 37°C for 5min, and the substrate 25-hydroxycholecalciferol (25-hydroxy[26(27)-*Me*-^{3}H]-cholecalciferol, 13.1 or 19.7Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) dissolved in 20μl of 98% (v/v) ethanol was then added to give a final concentration of 5nM except when indicated otherwise below under 'Results and discussion'. The reaction mixtures were flushed with 100% O_2 for 30–60s and incubated for 10min at 37°C, with gentle shaking. The reaction was stopped by the addition of 9ml of methanol–chloroform (2:1, v/v) and the mixtures were gently shaken overnight at 4°C. 25-Hydroxycholecalciferol and its metabolites were extracted by a slight modification of the method of Bligh & Dyer (1959). The extracts were chromatographed on columns (20cm × 1.5cm) containing 8.5g of Sephadex LH-20 (Pharmacia AB, Uppsala, Sweden). The metabolites were eluted with chloroform–*n*-hexane (13:7, v/v) and the eluates were collected directly into scintillation-counter vials. The radioactivity corresponding to each metabolite was measured by liquid-scintillation counting at constant efficiency (Holick & DeLuca, 1971; Galante *et al.*, 1972*a*).

Under the assay conditions described above, production of 1,25-dihydroxycholecalciferol was linear with time up to 20min, falling off rapidly beyond this until at 80min no further increase in the production was observed. The production of 1,25-dihydroxycholecalciferol was proportional to the homogenate concentration in the range 2–10% (w/v), and no enzyme activity was observed with homogenates that had previously been boiled for 20min. Formation of the dihydroxy metabolite was maximum between pH7.0 and 7.5.

In some experiments additions of Ca^{2+} (as $CaCl_2$), P_i [as a 1:4 (w/w) mixture of NaH_2PO_4 and Na_2HPO_4) and EGTA [ethanedioxybis(ethylamine)-*NNN'N'*-tetra-acetate] to the incubation mixtures were made to the final concentrations indicated below under 'Results and discussion'.

Preparation of cytoplasmic and particulate fractions. Chick kidneys were homogenized (approx. 10%, w/v) in 50mM-potassium phosphate buffer, pH7.5 at 4°C, and centrifuged at 1000$g_{av.}$ for 15min at 5°C,

The sediment was discarded and the supernatant re-centrifuged at 105000$g_{av.}$ for 60min at 5°C. The second supernatant was freeze-dried and stored at −70°C. The protein concentration was 1.74mg/100mg of dry powder. The ability of this fraction to bind 25-hydroxycholecalciferol was assayed by the method of Haddad & Chyu (1971).

In order to study the hydroxylase activities of particulate and cytoplasmic fractions, a 4% (w/v) homogenate of kidney tissue was prepared in 15mM-Tris – acetate buffer, pH7.4, supplemented with $MgCl_2$ (1.9mM), sodium succinate (5mM), sucrose (200mM), glucose 6-phosphate (5mM), $NADP^+$ (0.33mM) and glucose 6-phosphate dehydrogenase (600 units/l). The homogenate was centrifuged at 105000$g_{av.}$ for 60min. The supernatant was retained and the sediment was washed twice by resuspension in fresh medium and re-centrifugation. The rates of 1-hydroxylation of 25-hydroxycholecalciferol catalysed by the supernatant and particulate fractions separately, and by a reconstituted preparation made by resuspending the washed sediment in supernatant, were compared with that of the original homogenate.

Results and discussion

Increasing the concentration of Ca^{2+} in the assay medium produced a pronounced inhibition of 1-hydroxylation, which was virtually complete at 0.5mM-Ca^{2+} with a substrate concentration of 5nM (Fig. 1). Addition of the chelating agent EGTA enhanced the formation of 1,25-dihydroxycholecalciferol so that the maximum rate of production was obtained at a final concentration of EGTA of 0.2mM. The ability of low concentrations of EGTA to enhance hydroxylase activity is probably due to removal of free Ca^{2+} present in the homogenate. If this is the case, zero Ca^{2+} concentration would correspond to 0.2mM-EGTA, the point at which the maximum rate of hydroxylation was observed. Since 1mol of EGTA combines with 2mol of Ca^{2+}, the maximum effect of Ca^{2+} on the rate of production of the dihydroxy metabolite appears to occur over a concentration range of these ions of approx. 0–0.5mM. A decline in the rate of 1-hydroxylation was observed at EGTA concentrations greater than 0.2mM: this may represent the effect of chelation of metal ions other than Ca^{2+} that are essential to enzyme activity, e.g. Mg^{2+} (Fraser & Kodicek, 1970; Gray *et al.*, 1972).

In the absence of added Ca^{2+}, addition of increasing amounts of P_i, up to a final concentration of 20mM, had no effect on the rate of hydroxylation. However, when Ca^{2+} was present in an inhibitory concentration (0.2mM), P_i at concentrations above 8mM reversed this effect so that, in the presence of 20mM-P_i and 0.2mM-Ca^{2+}, a rate approaching that attained with no added Ca^{2+} was observed. P_i thus appears to prevent inhibition by Ca^{2+}, perhaps by lowering the effective Ca^{2+} concentration.

An apparent K_m of 1×10^{-7}M was calculated from the linear regression of the reciprocal of the rate of 1-hydroxylation on the reciprocal of the concentration of added 25-hydroxycholecalciferol, over the substrate concentration range 1–560nM. This value for K_m is significantly lower than that of 2.2×10^{-6}M reported by Gray *et al.* (1972) for the 1-hydroxylation reaction catalysed by isolated mitochondria. A possible explanation is that the K_m observed in homogenates does not correspond to the uptake of free substrate by the mitochondrial hydroxylase but to an earlier stage in the overall reaction. Proteins with the ability to bind metabolites of cholecalciferol have been identified in several vertebrate tissues and plasma (Edelstein *et al.*, 1972), and one such, present in the cytoplasm of rat kidney, has been used by Haddad & Chyu (1971) as the basis of an assay of 25-hydroxycholecalciferol. The possibility that a protein able to bind 25-hydroxycholecalciferol might occur in chick kidney homogenates was investigated, and the presence of a specific factor capable of binding 25-hydroxycholecalciferol, without catalysing its conversion into 1,25-dihydroxycholecalciferol, was demonstrated in the 105000$g_{av.}$ supernatant from chick kidney homogenates. A hyperbolic displacement

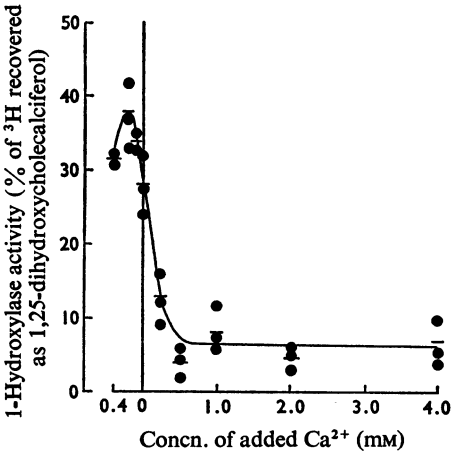

Fig. 1. *Inhibition of 25-hydroxycholecalciferol 1-hydroxylase activity of chick kidney homogenates by increasing concentrations of added Ca^{2+} in a final volume of 3ml*

Points (•) indicate replicate determinations and horizontal bars the mean values. Points to the left of the zero index on the abscissa were obtained in the presence of added EGTA at final concentrations up to 0.4mM.

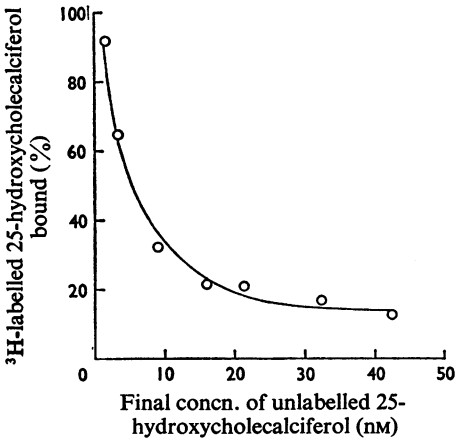

Fig. 2. *Displacement of ^{3}H-labelled 25-hydroxycholecalciferol from binding to cytoplasmic fraction by added unlabelled 25-hydroxycholecalciferol*

Increasing amounts of unlabelled 25-hydroxycholecalciferol (Roussel Laboratories; a gift from Dr. B. M. de Fossey, Paris, France) were added to fixed amounts of labelled 25-hydroxycholecalciferol and freeze-dried supernatant fraction (1–2mg) in a final volume of 1ml of 50mM-potassium phosphate buffer, pH7.5. Bound and free labelled 25-hydroxycholecalciferol were separated by adsorption of the free compound on dextran-coated charcoal after 60min at 25°C before the supernatant radioactivity was counted.

---

curve was obtained for the plot of percentage radioactivity bound versus concentration of unlabelled substrate (Fig. 2), and a linear plot of the data indicated an association constant of approx. 3×10^{-9}M.

More direct evidence for the participation of a cytoplasmic factor in the hydroxylation reaction was obtained from experiments on the separated and recombined particulate and cytoplasmic fractions.

When portions of the separated fractions and reconstituted preparations (equivalent in amounts to the original homogenate) were used, the rates of reaction (averages of four experiments) were respectively 3.6% (particulate fraction), zero (soluble fraction) and 29.5% (recombined preparation) of the rate with unfractionated homogenate. The substrate concentration was 125nM.

It is possible that some cytoplasmic factor other than that which binds 25-hydroxycholecalciferol may be responsible for the enhanced rate of 1-hydroxylation observed with the reconstituted preparation. However, the incubation medium used was shown in a separate experiment to be capable of supporting hydroxylation by much higher concentrations [equivalent to a 50% (w/v) homogenate] of a particulate fraction.

If the properties of the 25-hydroxycholecalciferol 1-hydroxylase system of chick kidney homogenates are shared by the intact system *in vivo*, the results of our experiments suggest two possible physiological mechanisms for the control of cholecalciferol metabolism. The first is the regulation of the hydroxylase activity by the intracellular concentration of Ca^{2+}, such that the rate of hydroxylation responds instantaneously and inversely to changes in the concentration of this ion. Intracellular Ca^{2+} concentration is in turn influenced by parathyroid hormone (Parsons & Robinson, 1971) and possibly by calcitonin (Parkinson & Radde, 1969). The maximum effect of Ca^{2+} on the rate of 1-hydroxylation is seen with this ion over the concentration range 0–0.5mM. The upper limit of this range is higher than the accepted range for the cytoplasmic concentration of Ca^{2+} of 0.1μM (Rasmussen, 1970). However, the site of the inhibitory action of Ca^{2+} on the hydroxylase system is not yet known, and inhibition may take place in an intracellular region, such as mitochondria, with a higher Ca^{2+} concentration than that of the whole cell. Fraser & Kodicek (1973) have concluded that direct control of 25-hydroxycholecalciferol 1-hydroxylase activity by Ca^{2+} is unlikely, since they failed to demonstrate a decrease in hydroxylation in kidney homogenates from vitamin D-deficient chicks after restoration of the serum calcium concentration to normal by the administration of cholecalciferol. However, this conclusion assumes that the serum calcium concentration accurately reflects the intracellular Ca^{2+} concentration.

The second potential regulatory factor, and one perhaps more suited to longer-term adaptation to altered metabolic needs, is the concentration of the presumed 25-hydroxycholecalciferol-binding protein in the cytoplasm. Fraser & Kodicek (1970) and Gray *et al.* (1972) have shown that the 1-hydroxylase system present in isolated mitochondria is able to form 1,25-dihydroxycholecalciferol from free 25-hydroxycholecalciferol, but that the K_m for this reaction is about 2×10^{-6}M (Gray *et al.*, 1972). If it is assumed that the K_m of the enzyme reflects its affinity for substrate, the affinity of the cytoplasmic binding protein for 25-hydroxycholecalciferol is 1000-fold greater than that of the hydroxylase. In the intact cell the 25-hydroxycholecalciferol 1-hydroxylase is believed to be located in mitochondria and 25-hydroxycholecalciferol reaches the cell from its site of production in the liver by way of the circulation. The effect of the binding protein may therefore be to accumulate 25-hydroxycholecalciferol, raising its concentration in the region of the enzyme and thus increasing the rate of product formation at concentrations that would be well below the K_m if the

free substrate were uniformly distributed throughout the intra- and extra-cellular fluids. For this hypothesis to be valid, either the protein-bound form of 25-hydroxycholecalciferol must be a substrate of the enzyme or some change that diminishes the affinity of the protein for 25-hydroxycholecalciferol in the vicinity of the enzyme must take place, since without some process of this nature it is difficult to see how the enzyme could compete successfully with the binding protein for the free substrate. If on the other hand the enzyme and binding protein act independently on free 25-hydroxycholecalciferol in homogenates, curved (convex-upwards) double-reciprocal plots of $1/v$ against $1/s$ should be observed, but such curvature was not detected in our experiments.

We thank the Medical Research Council and the Wellcome Trust for generous financial assistance.

Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–916

Edelstein, S., Lawson, D. E. M. & Kodicek, E. (1972) *Proc. 9th Eur. Symp. Calcified Tissues, Baden, in the press*

Fraser, D. R. & Kodicek, E. (1970) *Nature (London)* **228**, 764–765

Fraser, D. R. & Kodicek, E. (1973) *Nature (London) New Biol.* **241**, 163–166

Galante, L., MacAuley, S. J., Colston, K. W. & MacIntyre, I. (1972*a*) *Lancet* **i**, 985–988

Galante, L., Colston, K. W., Evans, I. M. A., MacAuley, S. J. & MacIntyre, I. (1972*b*) *Proc. 9th Eur. Symp. Calcified Tissues, Baden, in the press*

Gray, R. W., Omdahl, J. L., Ghazarian, J. G. & DeLuca, H. F. (1972) *J. Biol. Chem.* **247**, 7528–7532

Haddad, J. G. & Chyu, K. J. (1971) *J. Clin. Endocrinol.* **33**, 992–995

Holick, M. F. & DeLuca, H. F. (1971) *J. Lipid Res.* **12**, 460–465

Parkinson, D. K. & Radde, I. C. (1969) in *Calcitonin 1969, Proc. 2nd Int. Symp.* (Taylor, S. & Foster, G. V., eds.), pp. 466–471, Heinemann, London

Parsons, J. A. & Robinson, C. J. (1971) *Nature (London)* **230**, 581–583

Rasmussen, H. (1970) *Science* **170**, 404–412