2. Bacq, Z. M. and Harvey, A., Bull. Acad. Roy. Med. Belg. **17**, 13 (1952).

3. Gray, J. L., Moulden, E. J., Tew, J. T., and Jensen, H., Proc. Soc. Exptl. Biol. Med. **79**, 384 (1952).

4. Ahlquist, R. P., Am. J. Physiol. **153**, 588 (1948).

5. Selye, H., Veilleux, R., and Cantin, M., Science **133**, 44 (1961).

6. Schneyer, C. A., Am. J. Physiol. **203**, 232 (1962).

7. Schneyer, C. A., Finley, W. H., and Finley, S. C., Proc. Soc. Exptl. Biol. Med. **125**, 722 (1967).

8. Radley, J. M., Exptl. Cell Res. **48**, 679 (1968).

9. Barka, T., Exptl. Cell Res. **37**, 662 (1965).

10. Pohto, P. and Paasonen, M. K., Acta Pharmacol. Toxicol. **21**, 45 (1964).

11. Phillips, R. M., master's dissertation, Univ. of Alabama Med. Center, Birmingham, Alabama, 1966.

12. Greenspan, J. S., Melamed, M. R., and Pearse, A. G. E., J. Pathol. Bacteriol. **88**, 439 (1964).

13. English, J. A., J. Dental Res. **34**, 4(1955).

14. Cherry, C. P. and Gluckmann, A., Brit. J. Radiol. **32**, 596 (1959).

15. Ragins, H., Fried, M., Dombro, R., Dittbrenner, M., Liu, S. M., Johnson, L., and Sanfilippo, L. Am. J. Digest. Diseases **12**, 1256 (1967).

16. Barka, T., Exptl. Cell Res. **39**, 355 (1965).

17. Koller, P. C. and Smithers, D. M., Brit. J. Radiol. **19**, 89 (1946).

18. Lea, D. E., "Actions of Radiations on Living Cells." Macmillan, New York (1947).

Received Jan. 21, 1969. P.S.E.B.M., 1969, Vol. 131.

Ethanol-Induced Artifacts in the Metabolism of ^{3}H-Vitamin D_3* (33962)

G. PONCHON AND H. F. DELUCA

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

There has been much recent activity in the area of the early biochemical events following the administration of vitamin D to D-deficient animals (1). In such experiments, of major concern is the route and vehicle of administration. Although the oral route represents a natural or physiologic one, it leaves much to be desired because absorption of the vitamin is a slow, irregular and incomplete process (1–4). Intraperitoneal and intramuscular routes are neither direct nor physiological. Intravenous administration certainly appears to offer the best route for such studies, but the vehicle used represents the major concern. Early studies in this laboratory employed Tween-20 suspensions; however artifacts in the case of the apparent liver metabolites soon became obvious. Ethanol has since been used with apparent success; but this unphysiological vehicle has now revealed some artifactual effects. Abnormalities in the plasma disappearance of tritiated vitamin D_3 injected in ethanol solvent will be demonstrated by comparison with the fate of the same dose injected in a plasma vehicle.

Materials and Methods. [1, 2-^{3}H]-vitamin D_3 (sp act 24,453 dpm/IU or 0.44 μCi/μg) synthesized in this laboratory according to a method previously reported (5), was used. Radiochemical purity was assessed by UV spectrophotometry and biological assay (rat line-test) (6). The radioactive vitamin D_3 for injection was dissolved in absolute ethanol or in plasma from vitamin D-deficient rats at a concentration of 200 IU/ml. The plasma was obtained after centrifugation of heparinized blood (25 USP units of heparin/ml) from vitamin D-deficient rats. The [1, 2-^{3}H]-D_3 in 0.05 ml of absolute ethanol was added to not less than 2 ml of plasma. In such a preparation, the important dilution of ethanol (1:40) in plasma avoids apparent precipitation of the plasma proteins.

The actual concentration of each iv solution was checked in each series of experiments by measuring the radioactivity of three aliquots equivalent to the dose injected (0.05 ml) and delivered in counting vials by the same syringe and needle as used for the

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported by the USPHS Grant No. AMO 5800-08.

iv injection ("dose standards"). The radiochemical purity of the preparation was also routinely checked by column chromatography (5).

Holtzman male rats were fed for 2 months on the low vitamin D diet no. 11 (0.47% Ca, 0.30% P) (7) and averaged 150 g of body weight at the time of the experiments.

Twelve rats were injected intravenously under light ether anesthesia with 10 IU of [1, 2-^{3}H]-vitamin D_3 in 0.05 ml of ethanol (group I) or the same dose in 0.05 ml of plasma (group II). Blood samples (0.4 ml) from the tail vein were collected in small heparinized centrifuge tubes at specific time intervals after dosage and the plasma was separated by centrifugation. Hematocrits were performed periodically, using heparinized microhematocrit tubes (Yankee, Clay-Adams). Radioactivity measurements were performed on all plasma samples as described below. The proportion of the injected ^{3}H-vitamin D_3 in the plasma compartment was calculated accordingly. Total blood volume was estimated to represent 6% of the body weight at the first blood sampling. The remaining plasma volumes were calculated on the basis of the hemotocrit values and corrected for the blood losses during the experiments.

In some rats, 48-hr fecal collection and intestinal contents pooled together were ground in a mortar with 5% glucose to make a homogenous suspension of a known volume. Aliquots were measured for their tritium content.

The 0.1-ml plasma samples were pipetted into counting vials, dried and dissolved in 0.5 ml "N.C.S." solubilizer (Nuclear Chicago Corp.) (8) before mixing with 15 ml of toluene counting solution (2.0 g of PPO, 100 mg of dimethyl POPOP/liter of toluene A.R.). The "dose standards" consisting of 0.05 ml of ethanol solution of [1, 2-^{3}H]-vitamin D_3 were dried and directly dissolved in the toluene counting solution. The "dose standards" made of 0.05 ml of plasma solution of ^{3}H-vitamin D, were processed exactly as the plasma samples. A 0.5-ml aliquot of a known volume of homogeneously suspended feces was pipetted into cellophane combustion envelopes (Ivers-Lee Co., 215 Central Avenue, Newark, N.J.), lyophilized, and combusted according to the method of Kelly *et al.* (9). The resulting tritiated water was mixed with 20 ml of combustion counting solution (4.0 g of PPO, 50 mg of dimethyl POPOP, 200 ml of absolute ethanol and toluene A.R. ad 1 liter) (5). Except in the case of very small plasma samples, all radioactivity measurements were performed in triplicate with a Packard Tricarb 3003 liquid scintillation counter equipped with an automatic external standardization system 3951 (10). Counting efficiencies were in the order of 15–25% with the combustion counting solution, and of 25–35% with the toluene counting solution.

Results. As opposed to the injection of ethanol (group I), the use of 0.05 ml of plasma as an injection vehicle (group II) produced no indication of toxicity nor was there evidence of overt immunological incompatibility. The stability of [1, 2-^{3}H]-vitamin D_3 in plasma, at a concentration of 200 IU/ml is excellent when stored frozen at —10°. Repeated chromatography over a period of 3 months did not show any significant degradation of the vitamin. In connection with this it is interesting to recall that Thomas *et al.* (11) found no loss of antirachitic activity in sera frozen up to 1 year. The effect of plasma might be related to the specific protein binding the vitamin (11–13) resulting in protection of the groups susceptible to degradation.

Figures 1 and 2 summarize the plasma radioactivity pattern after injection of 10 IU of [1, 2-^{3}H]-vitamin D_3 in ethanol solution (group I) as compared to the plasma solution (group II). Although the patterns are quite similar 24 hr to 21 days after dosage (Fig. 1), they do differ during the first 12 hr (Fig. 2). The fraction of the dose present in the plasma of the animals receiving the plasma solution of labeled vitamin D is significantly higher, between 20 min and 6 hr, than in the plasma of rats receiving ethanol solution ($p<0.05$[1] at 20 min and 6 hr; <0.01 from 25 min to 4 hr). Both curves show, however, a

[1] Student's t test.

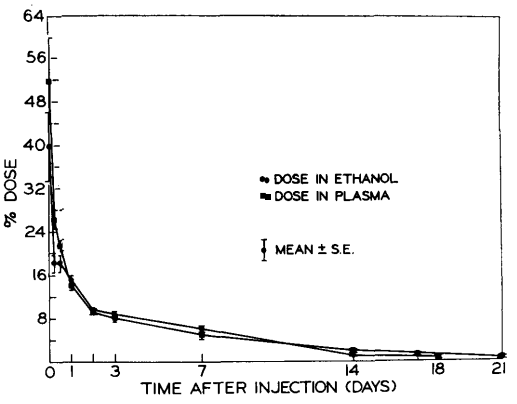

FIG. 1. Plasma radioactivity after iv injection of 10 IU of [1,2-^{3}H]-vitamin D_3 dissolved either in ethanol (group I) or in plasma (group II) to vitamin D-deficient rats (0–21 days).

similar overall pattern which was previously described as three successive phases (14).

The initial phase does not last longer than 30 min and consists of a very rapid disappearance of the plasma radioactivity. In the rats injected with ethanol solution of labeled vitamin D (group I), less than 40% of the dose may be recovered only 5 min after injection, and as much as 23% of the dose is further removed from the plasma during the remaining 25 min. The radioactivity does not disappear as quickly from the plasma of the rats of group II.

The rebound of plasma radioactivity (phase 2) which is evidenced in the group II between 1 and 4 hr after dose is barely seen

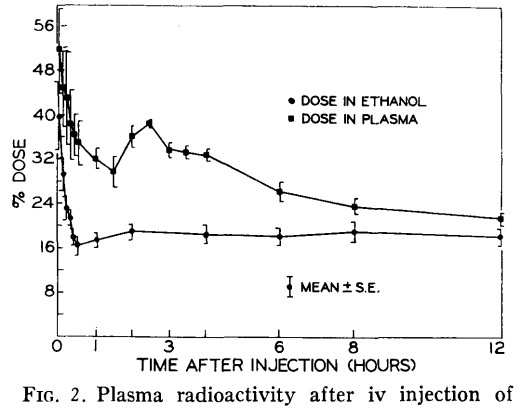

FIG. 2. Plasma radioactivity after iv injection of 10 IU of [1, 2-^{3}H]-vitamin D_3 dissolved either in ethanol (group I) or in plasma (group II) to vitamin D-deficient rats (0–12 hr).

in the rats injected with ^{3}H-vitamin D_3 in ethanol. The increase of plasma radioactivity during this period is only 2.6% over the low level at 30 min. However, the departure from the initial sharp slope remains striking. From the second day on and during the whole third phase of very slow decay, the 2 plasma radioactivity curves of groups I and II are no longer different.

The faster disappearance of plasma radioactivity in rats injected with the alcoholic solution of vitamin D_3 is reflected by an increase in their fecal excretion of tritium during the first 48 hr. Rats of group I excreted 22.7% ± 1.6 of the dose, while those of group II lost only 16.6% ± 2.4. These values represent the mean ± SE from 5 rats of group I and 4 rats of group II. However, due to rather large individual variations this difference in excretion is only indicative but not highly significant ($0.1 < p < 0.05$).

Discussion. Ethyl alcohol has been so far the most widely used vehicle for intravenous injection of labeled vitamin D in animal experiments (12, 13, 15) as well as in human investigation (16–18). This is indeed an excellent solvent for vitamin D, sterile and apparently not toxic when injected slowly in small quantity to humans. However, when it was used in rats, the toxic effects of ethanol soon became readily apparent despite the small amount injected (0.05 ml of absolute ethanol). The rats showed signs of neurologic intoxication such as somnolence and dizziness for approximately 2 hr after dosage. Significant hemolysis was visible in the plasma for 6 hr. A prolonged hemoglobinuria appeared, which might result from the hemol ysis or suggests kidney damage. These observations prompted us to investigate if ethanol would alter the metabolism of vitamin D, as compared to the results previously obtained with a more physiological vehicle, the plasma (14).

The present experiments revealed that the injection of ethanol does significantly accelerate the rate of disappearance of ^{3}H-vitamin D_3 from the plasma during the first 30 min after dose. As a consequence, the plasma level of radioactivity is lower in

ethanoltreated rats than when plasma is used as a vehicle, but the difference is no longer seen after 24 hr. In addition, the characteristic plasma rebound of radioactivity occurring between 1 and 4 hr (14) is markedly reduced. It appears, therefore, that ethanol is affecting the early metabolic events of vitamin D. These early events are the diffusion of the unchanged vitamin D_3 out of the plasma compartment and its uptake by various tissues, particularly the liver (first period). Part of the vitamin D_3 accumulated in the liver is hydroxylated into its biologically active metabolite, 25-hydroxycholecalciferol (19) and subsequently released into the blood, producing the characteristic rebound of the second phase (14, 19). It is easy to understand that any perturbation of these metabolic processes may have a profound influence on the fate of the injected vitamin. Not only the plasma and tissue distribution of the dose may be modified, but also the rate of conversion of vitamin D into its metabolites could be altered. The increased fecal excretion of tritium in ethanol-treated rats might reflect such a change.

How ethanol interacts with the metabolism of vitamin D is a matter of speculation. The faster disappearance of the dose from the plasma may be explained either by a decreased binding affinity of the plasma lipoproteins and specific carrier proteins (11–13) towards vitamin D, or by an increased cell membrane permeability. In favor of this second hypothesis is the phenomenon of hemolysis currently observed after ethanol injection, showing alteration of the red blood cell membrane. It is of interest that the rate of exchange of labeled cholesterol between human β-lipoproteins and erythrocytes is markedly increased by the addition of ethanol *in vitro*, but this has been observed at much higher concentrations of ethanol (10% v/v) (20). Another less likely possibility is that ethanol interferes directly with the intracellular metabolism of vitamin D in tissues, and particularly in the liver. It is well documented that the NAD^+:NADH ratio is decreased in the liver during ethanol oxidation. This has been demonstrated in liver slices (21), as well as *in vivo* after oral or intraperitoneal injection of ethanol to rats (22). Intraperitoneal injection of ethanol (3 g/kg of body wt) produces a highly significant accumulation of NADH in the liver as early as 30 min after dose. This effect vanished after 18 hr. A highly significant but transient decrease in $NADP^+$ content was also noticed after 60–90 min. Since the liver contains several steroid dehydrogenases utilizing NAD^+, and steroid hydroxylases requiring NADPH (23, 24), it is possible that the metabolism of vitamin D is affected by a change in these coenzymes, particularly its hydroxylation into 25-hydroxycholecalciferol (19). Changes in the proportion of hydroxy- to keto- forms of steroids in human blood was found 90 min after ingestion of ethanol (1 g/kg) (25). Although our rats did not receive more than 250–300 mg of ethanol/kg body of weight, the blood concentration achieved by intravenous injection is probably even higher than in the above cited experiments, and a similar metabolic effect on the liver is likely to occur.

Summary. The fate of vitamin D_3 in vitamin D-deficient rats was studied following the intravenous injection of 10 IU (0.25 μg) of [1, 2-^{3}H]-vitamin D_3 dissolved either in 0.05 ml of ethanol or 0.05 ml of blood plasma. Ethanol accelerates the disappearance of the radioactivity from the plasma compartment and decreases the characteristic rebound of plasma radioactivity occurring after 1–4 hr. The metabolic and toxic effects of ethanol are discussed, and the use of plasma as a vehicle for intravenous injections of vitamin D is advocated.

1. DeLuca, H. F., Vitamins and Hormones **25**, 315 (1967).

2. Callow, R. K., Kodicek, E., and Thompson, G. A., Proc. Roy. Soc. (London) Ser. B **164**, 1 (1966).

3. Schachter, D., Finkelstein, J. D., and Kowarski, S., J. Clin. Invest. **43**, 787 (1964).

4. Thompson, G. R., Lewis, B., and Booth, C. C., J. Clin. Invest. **45**, 94 (1966).

5. Neville, P. F. and DeLuca, H. F., Biochemistry **5**, 2201 (1966).

6. U.S. Pharmacopeia, Mack Printing Co., Easton, Pa., 15 ed., 889 (1955).

7. Guroff, G., DeLuca, H. F., and Steenbock, H., Am. J. Physiol. **204**, 833 (1963).

8. Hansen, D. L. and Bush, E. T., Anal. Biochem. **18**, 320 (1967).

9. Kelly, R. G., Peets, E. A., Gordon, S., and Buyske, D. A., Anal. Biochem. **2**, 267 (1961).

10. Dewachter, R. and Fiers, W., Anal. Biochem. **18**, 351 (1967).

11. Thomas, W. C., Jr., Morgan, H. G., Connor, T. B., Haddock, L., Bills, C. E., and Howard, J. E., J. Clin. Invest. **38**, 1078 (1959).

12. Chen, P. S., Jr. and Lane, K., Arch. Biochem. Biophys. **112**, 70 (1965).

13. Rikkers, H. R. and DeLuca, H. F., Am. J. Physiol. **213**, 380 (1967).

14. Ponchon, G. and DeLuca, H. F., J. Clin. Invest., in press.

15. Stohs, S. J. and DeLuca, H. F., Biochemistry **6**, 3338 (1967).

16. DeLuca, H. F., Lund, J., Rosenbloom, A., and Lobeck, C. C., J. Pediat. **70**, 828 (1967).

17. Avioli, L. V., Lee, S. W., McDonald, J. E., Lund, J. and DeLuca, H. F., J. Clin. Invest. **46**, 983 (1967).

18. Avioli, L. V., Williams, T. F., Lund, J., and DeLuca, H. F., J. Clin. Invest. **46**, 1907 (1967).

19. Ponchon, G., Kennan, A. L., and DeLuca, H. F., J. Clin. Invest., in press.

20. Bruckdorfer, K. R., Basford, J. M., and Green, C., Biochem. J. **100**, 33p (1966).

21. Smith, M. E. and Newman, H. W., J. Biol. Chem. **234**, 1544 (1959).

22. Slater, T. F., Sawyer, B. C., and Straüli, U. D., Biochem. J. **93**, 267 (1964).

23. Talalay, P., Ann. Rev. Biochem. **34**, 347 (1965).

24. Sih, C. J. and Whitlock, H. W., Jr., Ann. Rev. Biochem. **37**, 661 (1968).

25. Cronholm, T. and Sjövall, J., Biochim. Biophys. Acta **152**, 233 (1968).

Received Jan. 27, 1969. P.S.E.B.M., 1969, Vol. 131.

A Positive Correlation between Natriuresis and Inhibition of Renal Na–K–Adenosine Triphosphatase by Ouabain* (33963)

JERRY B. HOOK
(Introduced by W. D. Collings)

Department of Pharmacology, Michigan State University, East Lansing, Michigan 48823

A variety of natriuretic agents has been shown to selectively inhibit the Na–K-activated portion of the renal microsomal adenosine triphosphatase (ATPase) system *in vitro* (1–5). The data suggest that inhibition of this enzyme could be the mechanism whereby drugs inhibit renal sodium transport. Establishment of Na–K–ATPase as the renal receptor for natriuretic agents would be advanced if these drugs inhibited the enzyme when administered *in vivo*. Jones *et al.* (2) found that renal Na–K–ATPase from rats treated with diuretic mercurials was depressed. Hook and Williamson (6) obtained comparable results with furosemide and ethacrynic acid, even though ethacrynic acid did not produce natriuresis. However, in both studies the doses employed were sufficiently high that enzyme inhibition could have represented a toxicological, rather than pharmacological, effect. When smaller, diuretic doses of mercurials and ethacrynic acid were studied in dogs, Nechay *et al.* (5) could find no enzyme inhibition *in vitro* when the kidneys were removed and assayed. Thus, these authors could not implicate Na–K–ATPase as the renal receptor for these drugs.

The digitalis glycosides are powerful natriuretic compounds and are specific inhibitors of Na–K–ATPase *in vitro* (7). Both natriuresis and enzyme inhibition produced by digitalis can be reversed by addition of postassium [see Ref. (5)]. Furthermore, Palmer and Nechay (4) demonstrated that varying doses of ouabain, in the chicken, produced a biphasic effect on sodium transport that was mirrored by a similar biphasic effect on Na–K–ATPase when ouabain was

* Supported in part by USPHS grant 10913 and a grant from the Michigan Heart Association.