231. Isolation and Identification of Three Urinary Metabolites of Retinoic Acid in the Rat

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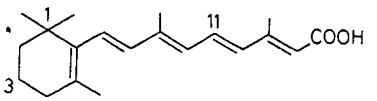
Summary. After the intraperitoneal administration of high doses of ^{14}C- and ^{3}H-labelled retinoic acid (**1**) to rats three major urinary metabolites have been isolated in microgram amounts by use of column, thin-layer and high-pressure liquid chromatography. Their structures were elucidated by mass spectroscopy and *Fourier* transform ^{1}H-NMR. spectroscopy as **2** (5-methyl-5-[2-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)vinyl]-2-tetrahydrofuranone), **3** (5-[2-(6-hydroxymethyl-2,6-dimethyl-3-oxo-1-cyclohexen-1-yl)vinyl]-5-methyl-2-tetrahydrofuranone) and **4** (6-(6-hydroxymethyl-2,6-dimethyl-3-oxo-1-cyclohexen-1-yl)-4-methyl-4-hexenoic acid). In these metabolites the tetraene side chain of **1** is shortened and the cyclohexene ring oxidized. The radioactivity of **2** and **3** accounted for about 10% (0.9% of the dose) each, metabolite **4** for about 6% (0.5% of the dose) of the total urinary radioactivity.

1. Introduction. – Retinoic acid (**1**), 3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid (*Scheme 1*), is the active principle of *Airol* ROCHE which is applied in acne therapy. It is also a potent growth-promotor in retinol-deficient rats [1]. However, retinoic acid is not like retinol a precursor of the retinal needed for the biosynthesis of visual pigments [2], although it has been found to be a normal metabolite of both retinal and retinol [3–6].

Scheme 1. *Retinoic acid*



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The search for metabolites of retinol or retinoic acid was intensified after there were suggestions that retinol and retinoic acid could be precursors of an unknown, active metabolite. After oral administration to rats, retinoic acid was rapidly excreted in the bile as retinoyl β-glucuronide [7]. The retinoic acid metabolite isolated from the livers of rats fed large doses of retinoic acid has been identified as 13-*cis*-retinoic acid [8]. Publications concerning unidentified metabolites in rat urine after intravenous or intraperitoneal administration of retinoic acid are numerous [6] [8–11]. As a result of experiments done with retinoic acid radioactively labelled in various positions *DeLuca & Roberts* suggested that the metabolites in the urine of rats had a shortened side chain [9] [12].

This paper describes the isolation, characterization and identification of three urinary metabolites after intraperitoneal administration of large doses of 15-[^{14}C]-retinoic acid and 10,11-[^{3}H]-retinoic acid to rats[2].

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[2] The configuration of the asymmetric centers of the metabolites **2**, **3**, and **4** has not yet been investigated.

2. Experimental. – *Radioactive Compounds.* The following labelled retinoic acids were available: 15-[^{14}C]-retinoic acid (59 μCi/mg) and 10,11-[^{3}H]-retinoic acid (273 μCi/mg).

Animal Experiment. To each of 36 male rats (*Füllinsdorf Albino SPF*, 380–420 g) 1 ml of a suspension of 1.07 g of retinoic acid, 9.9 mg 10,11-[^{3}H]-retinoic acid (2.7 mCi) and 20.3 mg of 15-[^{14}C]-retinoic acid (1.2 mCi) in 40 ml of a 1:2 mixture of Tween 80/NaCl 0.9% was administered intraperitoneally. The animals were kept in metabolism cages and received food once a day (Nafag 850/194) and water *ad libitum*. Urine and faeces were collected separately for 5 days.

Radioassay Procedure. Radioactivity was measured by liquid scintillation technique using a *Nuclear-Chicago* Mark I Instrument with external ^{133}Ba standard. The scintillation cocktail employed consisted of dioxane (350 ml), toluene (50 ml), ethylene glycol monomethyl ether (100 ml), naphthalene (40 g), and 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazol (Butyl-PBD, CIBA) (3.5 g).

Radioactive areas on thin-layer plates were eluted with ethanol before measurement. Eluates containing radioactive material from thin-layer chromatograms, high-pressure liquid chromatography or samples of urine were added directly to polyethylene counting vials containing 15 ml of scintillation medium.

Thin-Layer Chromatography (*TLC.*). Glass plates (20 × 20 cm) coated with silica gel 60 F 254 (0.5 mm) were purchased from *E. Merck* (Darmstadt). Prior to use they were washed with acetone and chloroform. The samples were applied to the plates in lines with a microdoser (*Desaga*). Solvent systems used in the development of the plates were: benzene/2-propanol 5:3, 4:1; hexane/acetone 7:3, 3:2; chloroform/acetone 3:2. UV.-light absorbing areas were scratched off and eluted with ethanol.

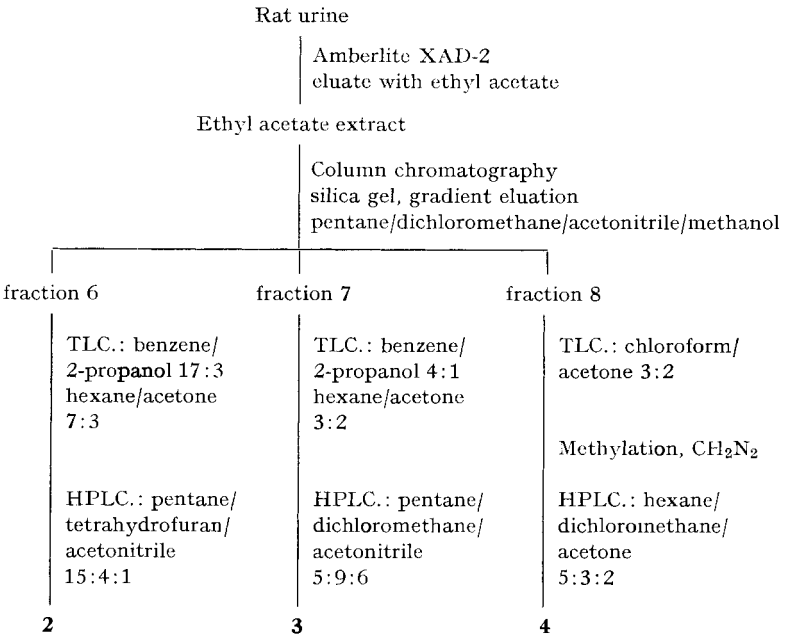
High-Pressure Liquid Chromatography (*HPLC.*). Pumping system: *Milton Roy* mini pump (24–240 ml/h, 5000 psi); pulse-damping: LDC 709 up to 1200 psi; detector: *Cecil CE 212* variable wavelength UV.-monitor with a 10 μl cell; column hardware: stainless steel tubing (internal diameter 3 mm), syringe injection port with septum; stationary phase: silica gel (Partisil 5, *Reeve Angel*), particle diameter 6 μ. Solvent systems: pentane/tetrahydrofuran/acetonitrile 15:4:1 or 5:9:6, hexane/dichloromethane/acetonitrile 5:3:2.

Mass Spectroscopy (*MS.*). All mass spectra were obtained with an *AEI*-MS 9 mass spectrometer using electron impact ionization, the high resolution data with an *AEI*-MS 902/DS 30 system. The samples were dissolved in 10 μl of methanol/dichloromethane 1:1, transferred onto the tip of a ceramic rod, and after evaporation of the solvent in a nitrogen stream introduced into the ion source of the mass spectrometer (source temp.: 250°, electron energy: 70 eV).

Proton Magnetic Resonance Spectroscopy (*^{1}H-NMR.*). The ^{1}H-NMR. spectra were run at 90 MHz on a *Bruker* HX 90/15 *Fourier* transform spectrometer equipped with a *Nicolet* 1083 computer. The interferograms were accumulated into 4 or 8 K of memory. Due to the small sample quantities available (as little as 4 μg) long-time accumulation of up to *ca.* 63 h (week end) was successfully applied (*ca.* 130000 scans) yielding excellent signal to noise ratios in these spectra. The samples were dissolved in less than 0.2 ml of $CDCl_3$ (so-called 100% D quality) obtained from *CEA-France* and *Stohler Isotope Chemicals*. Cylindrical NMR. micro tubes (*Wilmad Glas Company*, type 508-CP) were used. The D-signal of the solvent served as a field lock. The chemical shifts (in ppm) were referred to the signal of internal tetramethylsilane ($\delta_{TMS} = 0$ ppm).

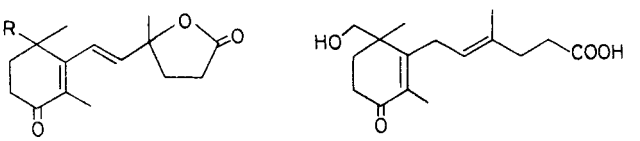
Isolation of the Urinary Metabolites **2**, **3** *and* **4**. The pooled urine of 36 rats collected for a period of 5 days was adjusted to pH 2 with 2 N HCl and chromatographed on a column (75 × 6.5 cm) of Amberlite XAD-2. After washing with 1.2 l of water a fraction of a mixture of unconjugated metabolites could be eluted with 2.4 l of ethyl acetate accounting for 69% of the urinary ^{3}H- and 61% of the urinary ^{14}C-activity. The eluate was further chromatographed on a column (60 × 5.5 cm) of silica gel 60 (*Merck*, 0.04–0.063 mm) using gradient elution (pentane/dichloromethane/acetonitrile/methanol). By this procedure three radioactive fractions (No. 6, 7 and 8) were isolated, containing about 40% of the ^{3}H- and 10% of the ^{14}C-activity. Upon TLC. with UV. and radioactivity detection and HPLC. with UV. detection the fractions 6, 7, and 8 yielded compound **2** (15 μg), compound **3** (37 μg), and compound **4** (60 μg), respectively. Compound **4** was methylated with diazomethane in ether for 3 min prior to HPLC. (*Scheme 2* and Fig. 2).

Scheme 2. *Isolation of the urinary metabolites* **2**, **3**, *and* **4** *of retinoic acid from rat urine*



3. Results. – In the urine excreted during the first five days following dosage the content of ^3H and ^{14}C accounted for 9 and 3.2% of the dose, respectively. No parent compound could be detected in the urine. The structures of the three metabolites of retinoic acid (**1**) isolated from rat urine are shown in *Scheme 3*. The metabolites **2**, **3**, and **4** contained only ^3H and no ^{14}C. Therefore it was obvious that the side chain was shortened by at least the last C-atom. Only **4** could be methylated by treatment with diazomethane. **2** and **3** had an UV. maximum at 260 nm (pentane/tetrahydrofuran/acetonitrile 15:4:1)corresponding to an $\alpha,\beta,\gamma,\delta$-unsaturated ketone. The structures of **2**, **3**, and **4** were deduced from mass and ^1H-NMR. spectra. They all have a C_{16}-skeleton with a side chain, shortened by 4 C-atoms compared to the parent compound, and an oxidized cyclohexene ring. In order to simplify the following

Scheme 3. *Urinary metabolites of retinoic acid* (% of total urinary radioactivity are given in parenthesis)



2 : R = CH_3 (10%)

3 : R = CH_2OH (10%)

4 (6%)

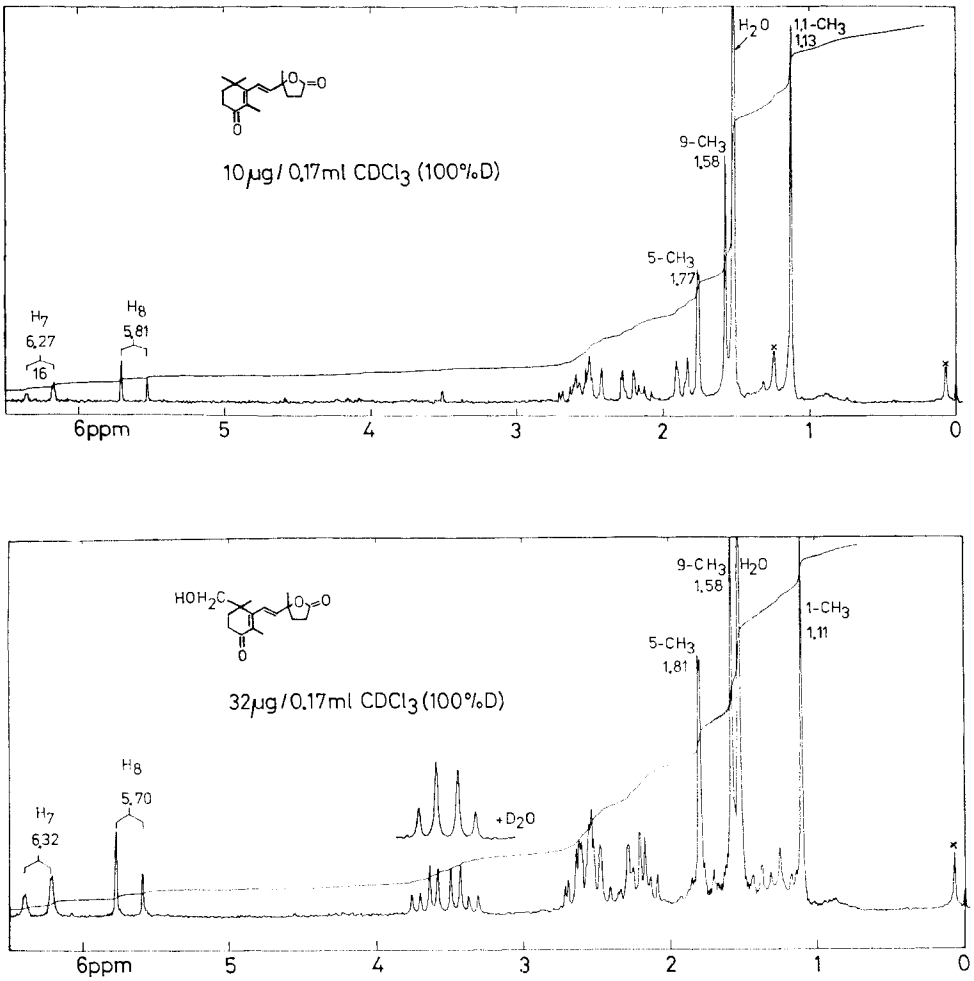


Fig. 1. *90 MHz* Fourier *Transform* ^{1}*H-NMR. spectra* (solvent signal not shown) *of* **2** (10 µg in 0.17 ml $CDCl_3$; 133000 pulses, 8 K) *and of* **3** (32 µg in 0.17 ml $CDCl_3$; 62000 pulses, 4 K). Insert: Addition of D_2O (4200 pulses, 4 K). Signals marked by asterisks are due to impurities

discussion of the spectroscopic data the numbering system given in *Scheme 1* is applied to all compounds.

Metabolite **2**. – The elemental composition of **2** is deduced from the mass spectrum as $C_{16}H_{22}O_3$. The fragmentation of the molecular ion is well compatible with the presence of the lactone residue: Cleavage of the C(8), C(9)-bond (probably after a H-shift from CH_3 at C(5) having made the C(8), C(9)-bond allylic) leads to two intense peaks at *m/e* 163 (base peak, $C_{11}H_{15}O$) and 99 (60% relative intensity, $C_5H_7O_2$).

The ^{1}H-NMR. spectrum of **2** (Fig. 1) reveals the presence of a *trans* -CH=CH– unit (*AB*-type spectrum of H-C(7) and H-C(8), $J_{AB} = 16$ Hz) with signals at 6.27 and 5.81 ppm, the former being slightly broadened by an additional small spin coupling. This is caused by the protons of the CH_3 at C(5), the signal of which appears

as a doublet ($J \sim 1$ Hz) at 1.77 ppm. The protons of the two geminal CH_3 at C(1) give rise to a singlet at 1.13 ppm, the CH_3 at C(9) is identified by its singlet at 1.58 ppm. The complex multiplets between *ca.* 1.8 and 2.7 ppm are assigned to the remaining CH_2– protons. Therefore the ^{1}H-NMR. data clearly proves the proposed structure **2** for this metabolite.

Metabolite **3**. – The molecular weight of **3** is deduced from the mass spectrum as 278. As in the mass spectrum of **2**, the presence of the lactone ring is indicated by an intense peak at *m/e* 99 (base peak). The complementary fragment, *m/e* 179, is of relatively low abundance (15%). The loss of CH_2O from the molecular ion, giving rise

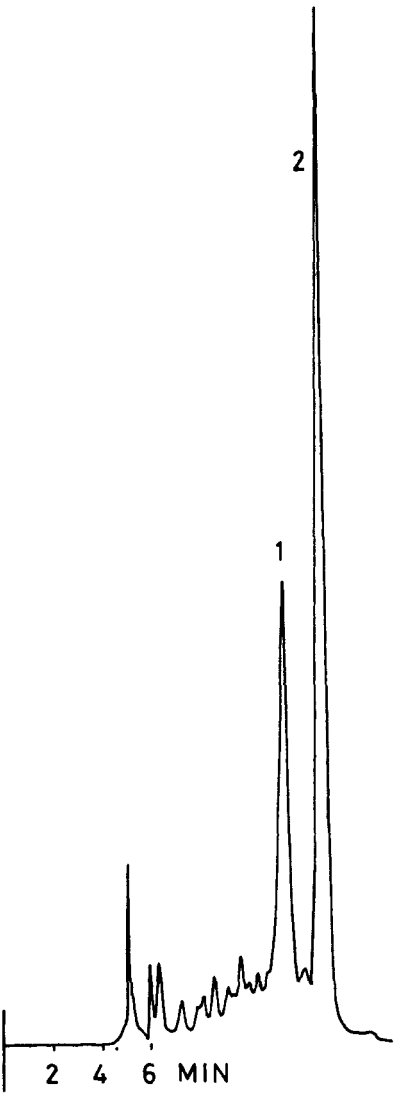


Fig. 2. *HPL-Chromatogram of fraction 6 purified by TLC.* Peak 2 represents 2 μg of metabolite **2**, peak 1 is due to an impurity. Solvent system: pentane/tetrahydrofuran/acetonitrile 25 : 4 : 1, flow: 0.53 ml/min, UV. detection at 260 nm

to a peak at *m/e* 248, not observed in the spectrum of **2**, reveals the presence of the hydroxymethyl moiety.

In the ^{1}H-NMR. spectrum of **3** (Fig. 1) the following relevant differences are observed in comparison to the spectrum of **2**: Obviously, instead of the two geminal methyl groups only one is left as seen from the intensity of the 1.11 ppm singlet (3 H). In addition, the newly appearing multiplet (*AB*-part of an *ABX*-type spectrum) around 3.5 ppm indicates the presence of a CH_2OH group with the methylen protons differently shielded due to the proximity of an asymmetric center. Upon addition of D_2O the spin coupling of OCH_2 with OH disappears. From these observations it follows that the hydroxymethyl group must be attached to C(1).

Metabolite **4**. – From the mass spectrum of the methyl ester of **4**, taken under high resolution conditions, molecular weight and elemental composition are deduced as 294 and $C_{17}H_{26}O_4$, respectively. As in the mass spectrum of **3**, the presence of a hydroxymethyl group is indicated by an intense peak (base peak) at *m/e* 264, due to the loss of CH_2O from the molecular ion.

The ^{1}H-NMR. spectrum of the methyl ester of **4** clearly indicates the presence of the same substituted cyclohexene ring as in **3** with relevant signals at 1.08 (3 H, H_3C–C(1)), 1.78 (3 H, H_3C–C(5)), *ca.* 2 to 2.6 (*ca.* 6 H, CH_2), and 3.37 and 3.72 ppm (2 H, OCH_2). In addition, the presence of a (CH_2CH=$C(CH_3)$)-unit is revealed by signals appearing at 2.95 (*d*, 2 H), 5.02 (*t*, 1 H), and 1.74 ppm (*s*, 3 H). These observations can be understood on the basis of structure **4** (the double bond in the side-chain is drawn in the *trans* configuration, although no proof can be derived from the spectroscopic data).

It should be pointed out that the ^{1}H-NMR. data given above would also be compatible, in principle, with a second structure having the double bond shifted from C(8), C(9) to C(9), C(10). The following experiments, however, strongly militate against this possibility. A sample of the methyl ester of **4** was deuteriated ($NaOCH_3$, CH_3OD, D_2O, 16 h, 20°). After neutralization with acetic acid, remethylation with diazomethane and purification using HPLC. the deuteriated methyl ester of **4** was investigated by MS. revealing the incorporation of a maximum of 4 D-atoms (15% D_4, 30% D_3, 40% D_2, and 15% D_1). The ^{1}H-NMR. spectrum, which was run from a sample of *ca.* 4 μg (total accumulation time 63 h) showed besides the disappearance of a part of the signals between 2 and 2.6 ppm, a significant change of the signal shape of the olefinic proton at 5.03 ppm which now appeared as a broad singlet rather than a triplet. This is interpreted by the assumption that molecular species with CD_2–CH= and CHD–CH= units were present which should give rise to superimposed broadened (by homoallylic coupling to the H_3C–C(9)) singlet and doublet signals, respectively.

Considering the mild deuteriation conditions, it can be safely assumed that deuterium was incorporated in the neighborhood of the 4-oxo group only, *i.e.* at C(3) and C(7) and not next to the acid group at C(11) (the ester group being hydrolysed during the reaction). Thus, the subunit in question, CH_2–CH=, whose ^{1}H-NMR. pattern has been modified by deuteriation, must contain C(7) and consequently formula **4** must be the correct choice.

4. Discussion. – The existence of a multiplicity of urinary retinoic acid metabolites has been published repeatedly [6] [9–11] [13] [14]. These metabolites were, in

part, characterized but not identified by MS. and ^{1}H–NMR. spectroscopy. In 1974 four urinary metabolites were isolated after injection of retinoic acid to rats and humans by *Rietz et al.* As they deduced from MS. and ^{1}H–NMR. spectroscopy the cyclohexene ring was oxidized to the cyclohexenone [15].

The metabolites **2**, **3**, and **4** described here have a C_{16}-skeleton lacking the 4 last C-atoms of the retinoic acid side-chain, and also a cyclohexenone moiety. *Sundaresan et al.* postulated the existence of six urinary metabolites after intraperitoneal injection of 6,7–[^{14}C]-retinoic acid into rats [16]. Of these the major metabolite was lacking at least the C(14) and C(15) of retinoic acid since it was not isolated when 15–[^{14}C]-or 14–[^{14}C]-retinoic acid was injected. This prompted these authors to suggest a metabolic attack on the hydrogen atoms at C(11) or C(12) [11].

Metabolite **4** perhaps represents the mysterious 'compound 5' isolated by *Yagishita et al.* after intraperitoneal administration of retinoic acid to rats [13]. They isolated two compounds from the intestine, 'compound 5' and 'compound 9'. 'Compound 5' was a carboxylic acid derived from retinoic acid by loss of at least the terminal C-atom (C(15)). Its UV. spectrum showed a peak at 252 nm, and the IR. spectrum had an OH-absorption band and two bands for keto-groups. 'Compound 9' could be a lactone similar to metabolites **2** and **3**.

The metabolites **2**, **3**, and **4** could derive from retinoic acid by the metabolic pathway postulated by *Roberts* & *DeLuca* in 1967 [9] [12]. As a result of experiments done with radioactive retinoic acid, labelled in various positions, *DeLuca* & *Roberts* suggested that the metabolic degradation of the retinoic acid side-chain starts with oxidation at C(14) and loss of the terminal C-atom (C(15)) by decarboxylation. This decarboxylation would lead to an acid with 19 C-atoms. In the experiments of *DeLuca* & *Roberts* about $^1/_3$ of the dose of retinoic acid was terminally decarboxylated. A further oxidation at position 12 of this C_{19}-acid would yield a β-keto acid. Since the β-keto acid would not be stable, loss of propionic acid could occur leading to intermediates with a skeleton of 16 C-atoms which would be direct precursors of the metabolites **2**, **3**, and **4**. It is still uncertain whether the metabolites **2**, **3**, and **4** are biologically active.

REFERENCES

[1] *J. F. Arens & D. A. van Dorp*, Nature *147*, 190 (1946).
[2] *J. E. Dowling & G. Wald*, Proc. nat. Acad. Sci. *46*, 587 (1960).
[3] *A. K. Bössaler & H. F. DeLuca*, Arch. Biochemistry Biophysics *142*, 371 (1971).
[4] *R. J. Emerick, M. Zile & H. F. DeLuca*, Biochem. J. *102*, 606 (1967).
[5] *D. S. Deshmukh, P. Malathi & J. Ganguly*, Biochim. biophys. Acta *107*, 120 (1965).
[6] *P. E. Dunagin, R. D. Zachman & J. A. Olson*, Biochim. biophys. Acta *124*, 71 (1966).
[7] *P. E. Dunagin, R. D. Zachman & J. A. Olson*, Science *148*, 86 (1965).
[8] *M. H. Zile, R. J. Emerick & H. F. DeLuca*, Biochim. biophys. Acta *141*, 639 (1967).
[9] *A. Roberts & H. F. DeLuca*, Biochem. J. *102*, 600 (1967).
[10] *P. R. Sundaresan & D. G. Therriault*, Biochim. biophys. Acta *158*, 92 (1968).
[11] *P. R. Sundaresan & G. M. Sundaresan*, Internat. J. Vit. Nutr. Res. *43*, 61 (1973).
[12] *H. F. DeLuca & A. Roberts*, Amer. J. Clin. Nutr. *22*, 945 (1969).
[13] *K. Yagishita, P. R. Sundaresan & G. Wolf*, Nature *203*, 410 (1964).
[14] *G. Wolf, S. E. Kahn & B. C. Johnson*, J. Amer. chem. Soc. *79*, 1208 (1957).
[15] *P. Rietz, O. Wiss & F. Weber*, Vitam. and Horm. *32*, 237 (1974).
[16] *P. R. Sundaresan, H. N. Bhagavan*, Biochem. J. *122*, 1 (1972).