References and Notes

1. M. H. Jones, *Science* 124, 442 (1956).
2. See "Principles of judgment," in J. P. Guilford, *Psychometric Methods* (McGraw-Hill, New York, ed. 2, 1954), chap. 12.
3. W. Landau and G. H. Bishop, *Arch. Neurol. and Psychiat.* 69, 490 (1953).
4. Tissues under progressive compression ischemia, pressure block, and so forth are *not* either perfect or dead. There is interference with normal physiological functioning, the nature of which is unknown.
5. R. S. Woodworth and H. Schlosberg, "Reaction time," in *Experimental Psychology* (Holt, New York, rev. ed., 1954), chap. 2.
6. P. P. Lele, D. C. Sinclair, G. Weddell, *J. Physiol. (London)* 123, 187 (1954).
7. J. W. Magladery, D. B. McDougal, Jr., J. Stoll, *Bull. Johns Hopkins Hosp.* 86, 291 (1950).
8. B. Frankenhaueser, *Acta Physiol. Scand.* 18, 75 (1949).

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An in vitro Effect of Vitamin D on Citrate Oxidation by Kidney Mitochondria

The possibility that an *in vitro* effect of vitamin D may be demonstrable appears evident from the effects on citrate metabolism which have been reviewed in recent publications (*1, 2*). In these it was reported that additions of vitamin D to rachitogenic and nonrachitogenic diets reduced the oxidation of citrate by kidney homogenates and mitochondria, thereby accounting for an increase in the citrate content of certain tissues and an increase in the citrate excretion in urine. Lately, we have succeeded in demonstrating a reduction in citrate oxidation by kidney mitochondria when vitamin D was added *in vitro*.

Young, male rats of the Sprague-Dawley strain were made vitamin-D deficient by the feeding of either a rachitogenic or a nonrachitogenic diet as described earlier (*1*). They were killed by decapitation, and kidney mitochondria were prepared, essentially by the method of Schneider (*3*).

The oxidations were carried out in a Warburg apparatus at 30°C with air as the gas phase. The incubation mixture, 3 ml in volume, contained 40 μmole of phosphate buffer (*p*H 7.3), 20 μmole of $MgCl_2$, 6 μmole of adenosine triphosphate, 0.08 μmole of cytochrome *c*, isotonic sucrose, and the indicated additions. Forty micromoles of glucose and excess hexokinase (Sigma) were added from the side arm of the flasks to prevent limitation of oxidation by a lack of phosphate acceptor (*4*). All substrates were added in amounts of 15 μmole, except citrate and succinate, which were added at 45 μmole, and oxalacetate, which was added at 10 μmole with an addition of 15 μmole of pyruvate.

When desired, the flask contents, prior to and following incubation, were deproteinized with 10-percent trichloroacetic acid. The filtrates, for the calculation of P/O ratios, were analyzed for P by the method of Lowry and Lopez (*5*). Citrate was determined by the method of Speck, Moulder, and Evans (*6*), and keto acids by the method of Friedmann and Haugen (*7*). Vitamin D and other compounds tested for their effect were added in propylene glycol or occasionally in the ethanol-serum albumin-phosphate buffer suspension of Nason and Lehman (*8*). In all cases, only 0.05 ml of each of these preparations was added to the contents of a flask. Control flasks received an equivalent amount of appropriate carrier.

Table 1. The *in vitro* effect of vitamin D on the oxidation of various substrates by kidney mitochondria.

Substrate	Without vitamin D (μl O_2)	With vitamin D* (μl O_2)	Decrease (%)
Citrate	85	32	62
Isocitrate	81	28	65
α-Ketoglutarate	75	80	0
Glutamate	69	55	20
Succinate	115	117	0
β-Hydroxybutyrate	35	31	11
Pyruvate plus oxalacetate	88	90	0

* One hundred and twenty-five micrograms of vitamin D_2 in 0.05 ml of propylene glycol per flask; other flasks received 0.05 ml of propylene glycol alone; 0.7 mg of mitochondrial nitrogen was added to each flask.

The comparative effect of vitamin D on the oxidation of various substrates (Table 1) clearly shows that vitamin D had a pronounced effect on citrate and isocitrate oxidation. Its effect on glutamate oxidation was small, but significant, while on the oxidation of α-ketoglutarate, succinate, β-hydroxybutyrate, and on pyruvate in the presence of oxalacetate, it had little or no effect. It is interesting to note that only the triphosphopyridine nucleotide systems are affected if one considers glutamate oxidized by both tri- and diphosphopyridine nucleotide pathways.

Table 2 illustrates the action of vitamin D in reducing citrate oxidation and α-ketoglutarate production while not affecting coupled phosphorylation efficiency to any degree.

In experiments not shown here, vitamins D_2 and D_3, when added *in vitro* in either of the carrier systems used, were equally active in reducing citrate oxidation, while equal quantities of 7-dehydrocholesterol, ergosterol, Δ^7-cholestenol, and cholesterol were inactive. However, it should be noted that in view of the different solubility characteristics of the sterols as compared with vitamin D, the possibility that they did not enter the mitochondria cannot be ruled out.

Table 2. The *in vitro* effect of vitamin D on the accumulation of α-ketoglutarate during citrate oxidation. The values represent an average of at least six determinations.

	Citrate oxidized (μg)	Oxygen consumed (μl O_2)	α-Ketoglutarate accumulated (μg)	P/O
Without vitamin D	630	83	14.4	3.0
With vitamin D_2*	180	46	8.5	2.7

* One hundred and twenty-five micrograms of vitamin D_2 in 0.05 ml of propylene glycol per flask; other flasks received only 0.05 ml of glycol; 0.7 mg of mitochondrial nitrogen was added per flask, and the oxidation was continued for 10 minutes.

The possibility that the resulting accumulation of citrate in kidney, and possibly in other tissues, may be an important factor in calcium transport and deposition appears increasingly alluring. Studies on these phases are in progress, especially with relation to specific enzyme systems.

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References and Notes

1. H. F. De Luca, F. C. Gran, H. Steenbock, *J. Biol. Chem.* 224, 201 (1957).
2. H. F. De Luca *et al.*, in preparation.
3. W. C. Schneider, *J. Biol. Chem.* 176, 259 (1948).
4. H. A. Lardy and H. Wellman, *ibid.* 195, 215 (1952).
5. O. H. Lowry and J. A. Lopez, *ibid.* 162, 421 (1946).
6. J. F. Speck, J. W. Moulder, E. A. Evans, Jr., *ibid.* 164, 119 (1946).
7. T. E. Friedmann and G. E. Haugen, *ibid.* 147, 415 (1943).
8. A. Nason and I. R. Lehman, *ibid.* 222, 511 (1956).

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Insect Nutrition and Metabolism of Sterols

The importance of cholesterol and related steroids in insect nutrition was first demonstrated in 1935 (*1*), and it is now well established that insects in general require a dietary sterol (*2*). The insects' nutritional requirement for sterols is indicative of an inability to synthesize the steroid nucleus, at least in physiologically adequate amounts. This characteristic is in direct contrast to that of the higher animals, in which steroids are apparently synthesized from simple com-

pounds such as acetate (*3*). The specificity of the insect requirement for sterols has been subjected to detailed investigation by a number of workers, and some of the important configurational requirements have been elucidated (*3*).

The metabolism of sterols by insects has been investigated much less intensively than have the nutritional aspects. Noland (*4*) postulated that certain nutritionally inadequate sterols could not be utilized by the insect because they inhibited the esterification necessary for assimilation from the intestinal tract. This hypothesis was not well supported by the results of a study of the structural specificity of sterol esterification *in vitro* by cockroach gut homogenates (*5*). Using larvae of *Callosobruchus chinensis* L. (cow pea weevil), Ishii (*6*) found that both tetrahydrostigmasterol and epicholestanol were nutritionally adequate for larval growth. Analyses of the sterols contained in the tissues of larvae that were fed on diets containing either of these unnatural compounds as the only dietary sterol indicated that the sterol nuclei had not been metabolically altered. Ishii concluded that the larvae were able to utilize tetrahydrostigmasterol without *in vivo* dehydrogenation at C_5 and C_6 and that they could utilize epicholestanol without isomerization of the hydroxy at C_3.

That an animal could be so loosely organized that a variety of sterols could be utilized at a cellular level without first being subjected to metabolic conversion into steroid compounds typical of the tissues seemed highly unlikely to us. A study was, therefore, undertaken of the effect of dietary sterols on tissue sterols in larvae of the confused flour beetle, *Tribolium confusum* Duval (*7*). In these experiments, the beetle larvae were reared from egg to larval maturity on synthetic diets to which known amounts of pure sterols had been added. The sterols used were cholesterol, 7-dehydrocholesterol, dihydrocholesterol, sitosterol, and ergosterol. The basal synthetic diet was composed of sterol-free fibrin, vitamin test casein, soluble starch, inorganic salts, and a mixture of ten B vitamins.

Before analysis, the larvae were held for 24 hours on a sterol-free diet to minimize interference from sterols contained in the gut contents. The larvae were then homogenized and extracted with a 1/1 mixture of acetone and absolute alcohol. Sterols were precipitated with digitonin. A modified Schoenheimer-Sperry reagent was used for color development, and absorbance after 1.5 and 33 minutes was measured at 620 mμ with a Beckman DU spectrophotometer. This procedure allows an estimation of those sterols which develop maximum color at 1.5 minutes (Δ5, 7) and of those that develop color maximum at about 33 minutes (Δ5) (*8*).

In the present study, 7-hydrocholesterol and cholesterol were used as standards for the "fast" and "slow" sterols, respectively. This analytic method does not permit estimation of sterols which either do not precipitate with digitonin or do not develop color with the Schoenheimer-Sperry reagent. In order to characterize further the "fast" and "slow" sterols found in the tissue extracts, and in order to detect sterols not detectable by the chemical method, a paper chromatographic method was employed. The method of McMahon *et al.* (*9*) was modified for this purpose. Whatman No. 1 filter paper was used, with antimony pentachloride in chloroform as the chromogenic agent. The method was standardized, employing 7-dehydrocholesterol, cholesterol, sitosterol, and dihydrocholesterol. With an ascending solvent mixture of 13 parts phenol, 30 parts methanol, and 57 parts water applied in one direction and a 14/45/41 mixture in the second direction, a two-dimensional chromatogram was obtained in which the several sterols were clearly separated.

Larvae grown on a natural diet (graham flour) and larvae grown on synthetic diets (each containing one of the pure sterols) were analyzed by both chemical and chromatographic methods. All experiments were replicated three times, and all analyses were run in duplicate. In every case, the principal tissue sterol present was a "fast" sterol which, by both its rate of color development with the Schoenheimer-Sperry reagent and its position on the paper chromatogram, was indistinguishable from 7-dehydrocholesterol. Regardless of the identity of the dietary sterol, this tissue sterol was present at concentrations between 850 and 900 μg/g of tissue. In all larval samples, a second sterol was also found, in amounts from 410 to 450 μg/g, and was chemically and chromatographically indistinguishable from cholesterol. No other tissue sterols were detected in amounts that permitted reasonably good identification. Chemical and chromatographic analyses of larvae from the different diets showed no differences attributable to the identity of the sterols in the diets.

Experiments were also set up in which beetle larvae were started on synthetic diets containing either no sterol or one of the nutritionally inadequate sterols, such as calciferol, progesterone, and testosterone. On these diets, however, no larval growth occurred, and none of the insects could be recovered and analyzed. Dihydrocholesterol diets did not promote optimum larval growth, although sufficient numbers of larvae were obtained to run the required analyses.

The results obtained in this study clearly indicate that *Tribolium confusum* larvae can metabolize dietary sterols to the extent of altering the side chain and the degree of saturation in the B ring of the nucleus. The nutritional adequacy of different steroid compounds is probably determined largely by the ability of the insect tissues to convert them into cholesterol and 7-dehydrocholesterol or compounds so closely related to these that they could not be distinguished from them by the methods employed in this study.

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References and Notes

1. R. P. Hobson, *Biochem. J.* (*London*) **29**, 1292 (1935).
2. H. Lipke and G. Fraenkel, *Ann. Rev. Entomol.* **1**, 17 (1956).
3. E. C. Albritton, *WADC Tech. Rept. No. 52* (1953); G. Fraenkel and M. Blewett, *Biochem. J.* (*London*) **37**, 692 (1943); H. McKennis, *J. Biol. Chem.* **167**, 645 (1947); and many others.
4. J. L. Noland, *Arch. Biochem. and Biophys.* **52**, 323 (1954).
5. J. E. Casida, S. D. Beck, M. J. Coles, *J. Biol. Chem.* **224**, 365 (1957).
6. S. Ishii, *Bull. Natl. Inst. Agr. Sci.* (*Japan*) **C5**, 29 (1955).
7. This study was supported in part by the National Institutes of Health, and has been approved for publication by the director of the Wisconsin Agricultural Experiment Station.
8. P. R. Moore and C. H. Baumann, *J. Biol. Chem.* **195**, 615 (1952).
9. J. M. McMahon, R. B. Davis, G. Kalnitsky, *Proc. Soc. Exptl. Biol. Med.* **75**, 799 (1950).

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Cytolysis versus Differentiation in Antineurula Serum

Complex antigenic mixtures derived from various developmental stages of *Rana pipiens* are being studied (*1*) in an analysis of the chemical patterns of ontogeny (*2*). Antigens were prepared from entire neurulae (Shumway stages 14 and 15) by a standardized homogenization in a Ten Broek tissue grinder with 0.05*M* phosphate-buffered saline at *p*H 7.4, and collection of the supernatants following centrifugation at 1500 *g* with final clearing at 10,000 *g* (*3*). Such supernatants, containing 1.6 to 2.0 mg of total N per milliliter, were emulsified (*4*), and quantities containing 0.2 ml of the antigenic mixture were injected under each scapula of four American chinchilla rabbits. Antisera were collected at periodic intervals and screened, using the interface precipitin reaction and the Ouchterlony agar diffusion technique (*5*). A representative antiserum, collected 4 months after injection, yielded a positive antigen dilution titer at 0.2 μg of N per milliliter. Similar titers were obtained with γ-globulin fractions prepared by a cold ethanol