uric acid. In fact, such an extrarenal mechanism may be a necessary prerequisite for these animals to take full advantage of the potentialities of uric acid excretion in water conservation. There is considerable evidence that water is reabsorbed in the cloaca of birds and reptiles (*2*). The urine produced in the kidney may have uric acid crystals suspended in it, but it must be relatively liquid in order to pass from the kidney through the thin ureters into the cloaca where water must be reabsorbed to produce the semisolid urine often observed in reptiles and birds. Experimental evidence, although not unequivocal, supports this concept. The removal of water from the cloacal contents could take place either through an active transport of water, or by the reabsorption of osmotically active substances with the water following passively through osmosis. If water were actively reabsorbed and cations such as sodium and potassium were to remain in the cloaca, the osmotic work involved would be very great (*3*) and would increase as the remaining fluid became more concentrated. If soluble cations were to remain in the cloaca they would finally form a highly concentrated solution, and it would be difficult to produce the almost dry urine pellets that are produced by reptiles and birds on a limited water intake.

The alternative hypothesis, that solutes are reabsorbed by active transport from the cloacal contents and that water follows passively is more plausible. The reptilian and bird urines produced by the kidney are always relatively dilute fluids. The reptilian kidney cannot produce urine more concentrated than the plasma, and the bird kidney in general can concentrate urine only to approximately twice the plasma concentration (the concentration limit for the mammalian kidney is approximately 17 times the plasma concentration). A simple improvement in the concentrating ability of the reptilian or bird kidney is probably not feasible because it would lead to the precipitation of so much uric acid in the kidney tubules that renal function would be impaired or blocked. The removal of a dilute or approximately isotonic fluid from the cloacal contents would be relatively inexpensive thermodynamically if the primary transport process were that of an active transport of cations with water following passively. If the same net result were achieved through an active transport of water, the expense would probably be several hundred times as great. In fact, in no case has it been convincingly demonstrated that the primary mechanism in the transportation of fluid is an active transport of water.

Should the removal of water from the cloacal contents be achieved through the reabsorption of cations, such as sodium or potassium, this would necessitate another route for the final elimination of these cations from the body. The nasal salt secreting gland is precisely such a mechanism.

It is probable that both sodium and potassium are reabsorbed in the cloaca under circumstances which require the withdrawal of water from the urine. The relative abundance of these two ions in the food varies, potassium being high in plant food and sodium more abundant in food of animal origin. Thus, the relative amounts of sodium and potassium to be eliminated vary with the diet, and this would explain why more potassium than sodium was eliminated by a plant-eating reptile such as the iguana. It also explains why the major anion in the nasal secretion of this animal was bicarbonate; plant food yields an excess amount of cation because the anions in the food, mainly organic acids, are metabolized and not excreted.

If water reabsorption depends on sodium reabsorption in the cloaca in connection with extrarenal excretion of the salt, we also have an explanation for the observation that, when a heavy salt load is given to a gull, the sodium content of cloacal urine often falls to very low values (*1*). This can better be understood if the response of the avian organism to an osmotic load is to achieve water conservation through an increased cloacal sodium reabsorption.

It has previously been suggested that the cloaca serves the function of sodium reabsorption (*4*). Evidence in favor of this opinion includes experiments in which the ureters of chickens were connected directly to the exterior so that the cloaca was bypassed. Such birds underwent excessive sodium loss but could be kept alive if sodium was added to the drinking water.

It is interesting to note that water conservation in the mammalian kidney is likewise achieved through an active transport of sodium from the urinary fluid to the blood, a transport which through the counter-current multiplier system permits an efficient withdrawal of water in the collecting ducts and thus the achievement of a high urine concentration (*5*). It now appears that the withdrawal of water from the urine of birds and reptiles may also be achieved through an active transport of sodium or potassium from the urinary fluid to the blood with water following passively. An extrarenal excretory mechanism is a necessity for the function of such a mechanism; it is possible that extrarenal excretion is a prerequisite for the evolution of birds and reptiles adapted to highly terrestrial habitats and that it is a prerequisite for these animals to utilize fully the advantage offered by the excretion of uric acid as the final metabolic end product of protein metabolism.

Obviously, water conservation is not always needed in a reptile or bird; at times they produce copious volumes of urine, and in this event there would be no need for the mechanism just presented (*6*).

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Synthesis of α-Linolenic Acid by Leishmania enriettii

Abstract. *The zooflagellate* Leishmania enriettii *synthesizes α-linolenic acid as determined by experiments with stearic acid uniformly labeled with* C^{14}. *This is the first demonstration of the synthesis of α-linolenic acid by a nonphotosynthetic organism.*

One of the few generalizations possible concerning the phylogenetic distribution of fatty acids is that higher plants synthesize α-linolenic acid (*cis, cis, cis*-9,12,15-octadecatrienoic acid) while higher animals do not (*1*). A further refinement of this statement resulted from the study by Erwin and Bloch (*2*) of the fatty acids of certain phytoflagellates. When grown in the light, *Euglena gracilis* contained α-

linolenic acid, most of which was in the chloroplasts. But *Euglena* grown in the dark (and therefore devoid of chlorophyll), and chlorophyll-less mutants of *Euglena* contained only insignificant amounts of α-linolenic acid. It appeared, therefore, that α-linolenic acid was characteristic of photosynthetic organisms containing chlorophyll. We have now found that the nonphotosynthetic zooflagellate *Leishmania enriettii* synthesizes α-linolenate. We conclude that there is no absolute correlation between chlorophyll-dependent photosynthesis and the synthesis of α-linolenic acid.

Leishmania and *Euglena* are both members of the Class Mastigiphora; the former being in the sub-Class Zoomastigina, the latter in the sub-Class Phytomastigina. That is, both organisms are flagellated protists, but one is "animal-like," containing no plastids; while the other is "plant-like," containing chloroplasts. The finding that the protozoan can also synthesize α-linolenic acid suggests that similar investigations of other Protista may reveal interesting taxonomic relationships.

Leishmania were grown as previously described (*3*) in Locke's solution over the blood-agar base of Senekjie. The cells were collected by low-speed centrifugation after filtration through gauze to remove pieces of agar. We washed the protozoa three times to remove soluble and particulate contaminants, and then extracted the lipids with chloroform-methanol. The extract was evaporated to dryness and the lipid dissolved in petroleum ether. We converted the fatty acids to their methyl esters either by transesterification in 0.5*N* methanolic NaOH, or by esterification, catalyzed by BF_3, in methanol after saponification and extraction of the fatty acids. The fatty acid composition was then determined by gas-liquid chromatography on ethylene glycol succinate polyester. Individual fatty acid methyl esters were isolated and purified by a combination of silicic acid chromatography of their mercuric acetate adducts and preparative gas-liquid chromatography. We have described all the methods mentioned in this paper in complete detail elsewhere (*4*). The fatty acids were identified as follows.

The methyl esters of the saturated fatty acids had the same retention times in gas-liquid chromatography as standards. They did not form mercuric acetate adducts, nor were their retention times altered by catalytic hydrogenation.

The dimethyl acetals of the fatty aldehydes had the same retention times as standards, did not form mercuric acetate adducts, and were not affected by hydrogenation. The acetals were stable to alkaline hydrolysis but converted to the free aldehydes by acid hydrolysis. The aldehydes were identified by gas-liquid chromatography and by oxidation to the corresponding fatty acids.

The monounsaturated and diunsaturated fatty acids were identified by the retention times of their methyl esters, by the chromatographic behavior on silicic acid of their mercuric acetate adducts, and by the retention times of the methyl esters of the saturated fatty acids produced by catalytic hydrogenation. Myristoleic acid and palmitoleic acid were not further characterized. Oxidative cleavage of methyl oleate by permanganate-periodate yielded pelargonic acid and azelaic acid as the only products, thus establishing the position of the double bond. Oxidative cleavage of methyl linoleate produced hexanoic acid and azelaic acid as the only products.

We initially identified α-linolenic acid by the retention time of its methyl ester, by the chromatographic behavior of its mercuric acetate adduct, and by its conversion to stearic acid upon hydrogenation. The positions of the double bonds were confirmed by oxidative cleavage. Azelaic acid was the only dicarboxylic acid formed. Propionic acid, the other expected product, was barely detectable, but no other monocarboxylic acid was found. In the method used, propionic acid is recovered poorly because of its water solubility, and the water solubility and volatility of methyl propionate. Therefore, we isolated C^{14}-labeled linolenic acid from *Leishmania* grown in the presence of stearic acid uniformly labeled with C^{14}. When this material was oxidatively cleaved, together with carrier oleate, linoleate and α-linolenate, propionic and azelaic acids were the only radioactive products.

The fatty acid composition of a typical culture of *Leishmania* is shown in Table 1. The most notable feature is the high percentage of α-linolenic acid. Other batches of cells had similar fatty acid compositions although the quantitative relationships varied somewhat. The fatty acid composition of the blood-agar medium (palmitate, 27 percent; palmitoleate, 4 percent; stearate, 15 percent; oleate, 15 percent; linoleate, 33 percent; α-linolenate, 3 percent; arachidonate, 6 percent), had no relationship to the composition of the fatty acids of the *Leishmania*. For example, the *Leishmania* contained 14 times more α-linolenic acid relative to linoleic acid than did the medium. Moreover, the cells contained 50 percent more α-linolenic acid than the blood-agar on which they were grown. Therefore, it seemed probable that *Leishmania* are capable of synthesizing α-linolenic acid.

To test this directly, we isolated and purified the individual fatty acids from the *Leishmania* grown in the presence of C^{14}-labeled stearic acid, and then determined the radioactivity of each acid.

Table 1. Fatty acid composition and distribution of radioactivity in *Leishmania enriettii* grown in the presence of C^{14}-labeled stearic acid. To each of five flasks containing 100 ml of medium (see text) 5 μc of C^{14}-labeled stearic acid (92 μc/μmole) were added as an albumin complex (2 μmole of C^{14}-labeled stearic acid per μmole of bovine serum albumin). The combined yield of cells was approximately 600 mg (wet weight). The fatty acids contained 4.7×10^6 count/min (10 percent of the added radioactivity). Individual fatty acids were separated and purified as described in the text. The concentration of each fatty acid was determined by analytical gas-liquid chromatography and its radioactivity was measured in a scintillation spectrometer using diphenyloxazole-toluene as scintillator-solvent. The distribution of radioactivity was calculated from the specific radioactivity of each isolated fatty acid and the percent composition of the original mixture. To confirm that the radioactivity was associated only with the indicated fatty acid, unlabeled mixed fatty acids were added to each purified fatty acid, the components of the mixture were separated by preparative gas-liquid chromatography, and each peak was collected on anthracene and counted. Contamination by other fatty acids could have accounted only for less than 0.5 percent of the radioactivity in stearaldehyde, for less than 0.1 percent of the radioactivity in oleic acid and linoleic acid, and for none of the radioactivity in α-linolenic acid. The precursor, C^{14}-labeled stearic acid, contained no detectable unsaturated fatty acids.

Fatty acid*	Composition (mass %)	Radioactivity (count/min)
Capric	1	0
Lauric	1	0
Myristic	8	0
Myristoleic	0.5	0
Palmitaldehyde	5	0
Palmitic	8	0
Palmitoleic	2	0
Stearaldehyde	1.5	0.5×10^5
Stearic	7	27×10^5
Oleic	22	9.2×10^5
Linoleic	17	4.6×10^5
α-Linolenic	23	4.6×10^5

* Fatty acids of chain length greater than 18 carbon atoms were not present in significant amounts. We did not determine fatty acids of chain length less than 10 carbon atoms. It is assumed that all double bonds are of the *cis* configuration.

From the results tabulated in Table 1, there can be no doubt that *Leishmania enriettii* is capable of synthesizing α-linolenic acid.

The absence of radioactivity in fatty acids of chain length less than 18 carbon atoms strongly suggests that the unsaturated 18-carbon fatty acids and stearaldehyde were synthesized from stearic acid without degradation of the carbon chain and reutilization of 2-carbon units.

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Lactate Dehydrogenase in Testis: Dissociation and Recombination of Subunits

Abstract. *Electrophoretic resolution of lactate dehydrogenase in mature testes from a variety of animals revealed one or more unusual isozymes in addition to the usual five forms. Dissociation of the enzyme and recombination of the polypeptide subunits led to the formation of new isozymes and to a redistribution of activity among those normally present, indicating that lactate dehydrogenase synthesis in postpubertal testis is controlled by more than two genes.*

Five types of lactate dehydrogenase (LDH) have been identified in mammalian tissues by the method of starch-gel electrophoresis (*1*), LDH-1 being nearest the anode, and LDH-5 nearest the cathode. Evidence now available (*2*) suggests that each isozyme is composed of four polypeptide chains which are assembled from two different polypeptide units, A and B. Thus, isozymes 1–5 have the following polypeptide composition: A^0B^4, A^1B^3, A^2B^2, A^3B^1, and A^4B^0. If the synthesis of the A and B polypeptides is controlled by two different genes, then the isozymic repertory of any tissue or cell would depend on the relative activity of these two genes. Also it is evident that the maximal number of isozymes in any tissue would be limited to five. Our finding of six or more LDH isozymes in mature testes must, therefore, be related to the activity of additional genes.

Tissues were obtained immediately after death, except for human tissues which were obtained not later than 24 hours after death. Washed rabbit sperm were prepared from fresh ejaculates; bull sperm were concentrated from epididymes. Methods for preparation and electrophoresis of tissue homogenates and for localization of LDH isozymes in starch gel were those previously described (*3*). Dissociation of the LDH isozymes was accomplished by adding sodium chloride and phosphate, *p*H 7.0, to fresh homogenates in final concentrations of 0.5*M* and 0.1*M*, respectively (*4*). These mixtures were stored at a temperature of −20°C for 24 hours, thawed, and immediately subjected to electrophoresis. Measurement of LDH in these preparations with lactate as substrate revealed no loss of activity.

Analysis of LDH isozymes in testes from man, rabbit, dog, and mouse revealed one unique band ("band X") in addition to the usual ones (*5*). In the samples from man the electrophoretic position of "band X" was between LDH-3 and -4, in those from rabbit and dog, between LDH-4 and -5, and in mouse, behind LDH-5. Two bands of LDH "X" activity were seen in homogenates of guinea pig and rat testes, one between LDH-3 and -4, and the other between LDH-4 and -5. Preparations from bull testes exhibited three "band X" isozymes, one between LDH-3 and -4, and two between LDH-4 and -5. The positions of LDH isozymes in duck, chicken, hog, and cat testes corresponded to those of the isozymes in other tissues, and no electrophoretically distinct forms were evident.

To determine the relative amounts of the isozymes in mature human testes, each isozyme was eluted from starch and LDH activity was measured simultaneously with $2 \times 10^{-4}M$ pyruvate and reduced nicotinamide adenine dinucleotide ($NADH_2$) in one assay, and $2 \times 10^{-1}M$ lactate and nicotinamide adenine dinucleotide (NAD) in the other. "Band X" isozyme represented 11 percent of the total activity with pyruvate as substrate, and 12 percent with lactate as substrate. The individual contributions of LDH-1, -2, -3, -4, and -5 to total LDH activity were 12, 29, 36, 8, and 4 percent with pyruvate and 6, 27, 44, 8, and 3 percent with lactate.

Approximately 80 percent of the LDH activity in washed human, rabbit, and bull sperm resided in the "band X" area. Also in a series of 40 rabbits of different maturity "band X" activity did not appear until the onset of spermatogenesis at the 8th week of life. These observations suggest that "band X" material may play a significant role in sperm function.

In addition to the characteristic electrophoretic behavior of the "X" bands some, but not all, demonstrated unusual activity with α-hydroxy acids and with certain analogs of NAD. Allen (*6*) was the first to show that mouse testis and epididymis contained an isozyme which demonstrated greater activity with (DL) α-hydroxybutyrate and (DL) α-hydroxyvalerate than did the other isozymes. Since similar results were obtained with "band X" LDH from the mouse in this study, it appears that the unusual isozyme described by Allen is indeed "band X". The "X" bands of rat preparations demonstrated high activity with (DL) α-hydroxyvalerate. However, the "X" bands from man, bull, dog, guinea pig, and rabbit did not react with such α-hydroxy acids. As previously reported (*3, 7*), there is a high ratio of acetylpyridine adenine dinucleotide to NAD activity for "band X" from human testes and sperm. This was not observed in the other testes used in this study.

The unusual electrophoretic characteristics of "band X" from all sources studied and the unique catalytic properties of some raised the question whether these enzymes had been improperly classified as isozymes of LDH. In washed human, rabbit, and bull sperm approximately 80 percent of the conversion of lactate to pyruvate is accomplished by the "band X" enzyme, indicating that "band X" is the major form of LDH in sperm from these species. Further evidence for classifying "band X" as an LDH isozyme was obtained by dissociating and reassociating LDH in homogenates prepared from mature testes exhibiting "band X" activity. The results observed in human and rabbit testes are shown in Fig. 1. The untreated homogenate of human testis exhibits six major areas of LDH activity. After dissociation and reas-