tubule. There was similar reabsorption in *Necturus* (*6*). In view of the widespread use of inulin as a reference for water movement into and out of the renal tubule, it seemed important to clarify the question of inulin reabsorption.

We have carried out experiments different from those of Shehadeh *et al.* (*5*) and Scott *et al.* (*6*), using free-flow microinjection (*7*). The experiments were based on the assumption that inulin injected into a tubule in one kidney would be detected in the urine from the contralateral kidney if it were reabsorbed into the circulation. We used 18 male Wistar rats and anesthetized them by intraperitoneal injection of 50 mg of pentobarbital per kilogram of body weight. The left kidney was exposed for microinjection through an abdominal incision, and both ureters were catheterized with polyethylene tubing. Inulin-carboxyl-C^{14} or inulin-methoxy-H^3 (*8*) in isotonic saline colored with nigrosine was injected by micropuncture into proximal tubules over a period of 40 to 200 seconds. The injected tubule was not blocked with oil, and the rate of inulin infusion into the tubule was continuously adjusted to prevent backflow toward the glomerulus. Experiments were performed both during intravenous infusion of hypertonic sodium chloride solution (2.5 percent) and in nondiuretic rats in order to detect inulin reabsorption if it occurred only when the rate of urine flow was slow. Urine was collected over periods of 5 to 10 minutes for 40 to 75 minutes. To shorten the collecting period required in nondiuretic rats—the period was long due primarily to the dead space of the ureteral catheters—0.2 to 0.3 ml of 20 percent mannitol solution was generally administered intravenously 7 to 15 minutes after the injection of inulin into the tubule. This interval was sufficient for passage of the injected inulin through the nephron, so that the mannitol only flushed the urine from the renal pelvis and catheter into the collecting vial. Reabsorbed inulin was estimated as twice the amount in the urine from the contralateral kidney. At the end of the experiment puncture sites were localized by microdissection (*4*).

Table 1. Inulin reabsorption after microinjection into proximal tubules in the rat.

Site of injection (% of proximal tubule)	Inulin reabsorbed* (%)	Urine flow rate (μl/kg min)	Isotope
Intravenous infusion of 2.5 percent NaCl			
20	0	90	H^3
20	1.4	100	C^{14}
22	0	55	C^{14}
25	1.5	50	C^{14}
26	0	70	C^{14}
27	0	100	C^{14}
27	2.4	90	H^3
28	0.3	110	H^3
30	0	80	H^3
31	0	70	C^{14}
34	0	120	H^3
40	2.4	40	C^{14}
42	0	60	C^{14}
44	0.8	75	C^{14}
45	0	40	C^{14}
44	0.5	120	C^{14}
46	0.4	70	H^3
48	0.4	60	H^3
56	0	110	C^{14}
Nondiuretic rats			
23	0		H^3
23	0.7		H^3
26	0		H^3
28	1.4		H^3
30	0.6		H^3
33	0.6		H^3
34	1.8		C^{14}
38	0		H^3
39	0.8		H^3
46	0		H^3
46	2.0		H^3
47	1.6		H^3
48	0.4		H^3
50	1.2		H^3
51	0.6		C^{14}

* The reabsorbed inulin is calculated as follows: (2 × inulin activity in urine from right kidney/total inulin activity recovered) × 100. Where reabsorption is indicated as 0, radioactivity in the urine from the right kidney was less than 2 standard deviations above background level.

The results (Table 1) show that there was little or no reabsorption of inulin-C^{14} or inulin-H^3 (*8*) under these conditions. Further, the physiological significance of the small reabsorption in certain experiments seems highly questionable. There was no correlation evident between the percentage of reabsorption and the site of injection along the proximal tubule. Excretion by the contralateral kidney may well have resulted from unnoticed small droplets of inulin inadvertently left on the surface during micropuncture; inulin droplets deliberately deposited on the intact capsule of four kidneys were rapidly absorbed into the circulation, as evidenced by the prompt appearance of inulin in the urine from both kidneys at an equal rate. Conceivably, also, stretching of the tubule by too vigorous injection may lead to reabsorption by mechanical damage or direct injection into peritubular capillaries. Our results agree with those obtained on animals that had profuse osmotic diuresis (*7*), but are at variance with those reported by Shehadeh *et al.* (*5*). We conclude that inulin is a satisfactory marker for quantitative measurement of water reabsorption from the renal tubule of the rat under conditions of free flow.

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9. Supported by grant-in-aid from the American Heart Association and PHS grant H-02334. Y.G., on leave from the Department of Pharmacology, School of Medicine, Jerusalem, Israel, is supported by an NIH International Postdoctoral Research Fellowship. C.W.G. is a Career Investigator, American Heart Association. W.E.L. is an Established Investigator, American Heart Association, and Markle Scholar in Academic Medicine.

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Isolation and Characterization of DNA from Kinetoplasts of Leishmania enriettii

Abstract. *The DNA of* Leishmania enriettii *can be separated by equilibrium sedimentation in cesium chloride into a major band of density 1.721 and a minor component of density 1.699. DNA from isolated kinetoplasts of this protozoan was identified as the less dense minor component.*

Within the protozoan family Trypanosomatidae an unusual organelle called the kinetoplast is situated in close proximity, if not actually connected, to the proximal end of the flagellum. The kinetoplast contains a substantial quantity of DNA and in part resembles a mitochondrion (*1*). Kinetoplast DNA has been isolated and found to differ in density from the nuclear DNA when sedimented to equilibrium in cesium chloride.

Leishmania enriettii was cultivated on Senekjie's medium (*2*) for 4 to 5 days and harvested by horizontal cen-

trifugation at room temperature in 250-ml bottles with conical bottoms for 25 minutes at 1500*g*. Pellets of protozoa were washed in Locke's solution, then in distilled water.

Total DNA was extracted according to the method of Marmur (*3*). Pellets were lysed with a solution of 0.015*M* NaCl and 0.0015*M* sodium citrate containing 0.01*M* ethylenediaminetetraacetate and 0.5 percent sodium lauryl sulfate (*p*H 7.5), then mixed with 5*M* sodium perchlorate to make the solution 1*M* and deproteinized twice with amyl alcohol–chloroform. Nucleic acid was precipitated with 2 volumes of carefully layered 95 percent ethanol, and the DNA was collected by wrapping on glass rods.

Kinetoplast DNA was isolated by a different procedure. Suspensions of organisms, washed as already described, were diluted with 10 volumes of distilled water and kept at room temperature for 2 to 4 hours, during which time many organisms were ruptured by osmosis. Intact organisms were removed by centrifugation for 5 minutes at about 50*g*. Ruptured organisms stained with acridine orange (*p*H 6.8, 10 μg/ml) were observed to have ruptured nuclei, as evidenced by a diffuse yellow-green fluorescence throughout, except for brightly fluorescent, yellow-green kinetoplasts. The nonkinetoplast DNA was largely digested and removed by adding deoxyribonuclease (50 μg/ml of suspension) for 10 to 20 minutes at room temperature. After this procedure the protozoa appeared as ghosts and the brilliant yellow-green fluorescence of the kinetoplasts remained undiminished while relatively little "nuclear" fluorescence could be observed. Satisfactory preparations were washed once with 0.15*M* NaCl containing 0.1*M* ethylenediaminetetraacetate to inactivate the deoxyribonuclease, and pellets were collected by centrifuging for 5 minutes at about 1500*g*. The DNA of the resulting pellets was extracted as described before except that the protein-containing interphase of the amyl alcohol–chloroform step was further extracted with a solution containing 0.015*M* NaCl and 0.0015*M* sodium citrate. The two supernatants were combined and precipitated with alcohol. Insufficient DNA was present to allow collection on a glass rod, but the precipitate was recovered by centrifugation for 15 minutes at about 1500*g*. The pellet was dissolved in a solution containing 0.015*M* NaCl and 0.0015*M* sodium citrate. Its absorption spectrum was that of a nucleic acid. This solution was then treated at 37°C for 30 minutes with previously heated ribonuclease, 50 μg/ml in a solution containing 0.015*M* NaCl and 0.0015*M* sodium citrate, reprecipitated with 95 percent ethanol, and centrifuged into a pellet. The pellet was suspended in 3 ml of a solution containing 0.015*M* NaCl and 0.0015*M* sodium citrate and quantitated by its absorption spectrum in ultraviolet light. Solutions of kinetoplast DNA were stored for short periods in a refrigerator or, for long periods, were kept refrigerated in 95 percent ethanol.

Analytical ultracentrifugation in cesium chloride equilibrium density gradients (44,770 rev/min, 20 to 24 hours) revealed the presence of two distinct bands in DNA extracted from whole *Leishmania*: a major component of relative density 1.721, and a minor component of relative density 1.699 (*4*) (Fig. 1). The minor band consistently came to equilibrium much more rapidly than either *Escherichia coli* DNA or the major band; the minor band was visible as a distinct band after centrifugation for 64 to 128 minutes at 44,770 rev/min (Fig. 2). This characteristic, as well as the position of the minor band relative to *E. coli* DNA used as a marker, facilitated the identification of this minor component. It was the only component present in the preparations of kinetoplast DNA (Fig. 3). As calculated from the equilibrium density characteristics, the guanine-cytosine content of the major band was 57 percent, that of the minor band, 36 percent.

When DNA from whole *Leishmania* was treated with deoxyribonuclease, neither band was observed to form after 20 hours of centrifugation (Fig. 4). In another experiment, kinetoplast DNA and *E. coli* DNA were treated with deoxyribonuclease in cesium chloride after separation of the two bands by equilibrium sedimentation. In this case also, neither band re-formed after 20 hours of centrifugation. All of the solutions of DNA in cesium chloride contained a variable quantity of material which absorbed ultraviolet light, sedimented rapidly to the bottom of the cell, and subsequently slowly "floated" upward (Fig. 2). This material consisted in part, if not solely,

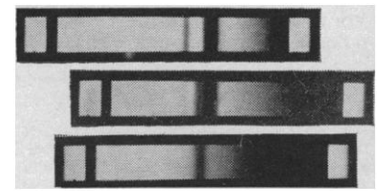

Fig. 1. (Top) DNA from whole *Leishmania*; (middle) DNA from whole *Leishmania* plus *E. coli* DNA; (bottom) *E. coli* DNA. All photographs oriented so that the bottom of the cell is seen on the right.

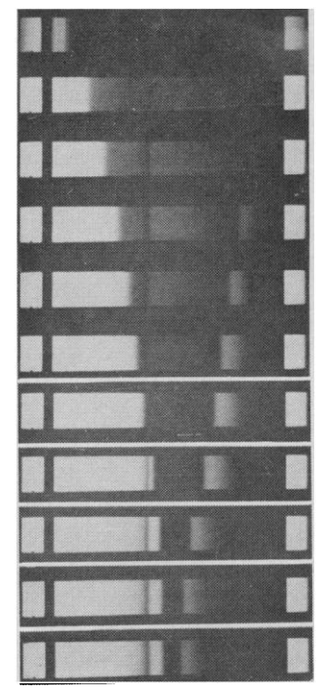

Fig. 2. From top to bottom, DNA from whole *Leishmania* after 0, 1, 2, 3, 4, 5, 7, 9, 13, 17, and 21 "hours" of centrifugation. An "hour" is actually 64 minutes.

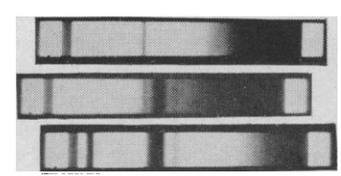

Fig. 3. (Top) Kinetoplast DNA; (middle) kinetoplast DNA plus *E. coli* DNA; (bottom) *E. coli* DNA which revealed a small band heavier than the bulk of the bacterial DNA.

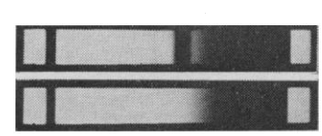

Fig. 4. (Top) DNA from whole *Leishmania*; (bottom) same preparation after incubation with deoxyribonuclease.

of a contaminant of the cesium chloride. The contaminant persisted after treatment of the solution with activated charcoal and recrystallization of the salt. It was relatively insoluble and showed yellow fluorescence when examined under ultraviolet light.

Our results establish that there are two categories of DNA in *Leishmania* and indicate that the small quantity of the less-dense band of DNA is located in the kinetoplast. It is not possible to state categorically that no minor component exists in the nucleus. However, it would appear that little or no major component is present in the kinetoplast. The rapid equilibration of the minor component suggests an unusual configuration or aggregation such that it responds to the centrifugal force more rapidly than the DNA of the nucleus and *E. coli*. In view of this behavior, the guanine-cytosine content of the minor band might not be 36 percent as calculated from the apparent density, 1.699, by the equation of Sueoka *et al.* (*5*).

To date, three pieces of evidence support the contention that the minor DNA component is of lower molecular weight than the major DNA component and that its rapid banding results from its having a more favorable shape factor than the major component. (i) When both types of DNA were sheared by sonication (*6*), as evidenced by extremely slow formation of broad bands, both broad bands were found at the same density as their intact counterparts. (ii) After being equilibrated in cesium chloride the rotor was stopped for 3 hours and then started again; despite the rotation of the cell contents from a vertical to a horizontal position upon stopping the rotor, when the rotor again reached 44,770 rev/min the major band was observed in its equilibrium position, although now about twice its equilibrium breadth. The minor band had vanished, presumably because of diffusion or mixing, or both, but it re-formed within an hour in its usual fashion. (iii) When whole *Leishmania* DNA dissolved in a solution containing 0.015*M* NaCl and 0.0015*M* sodium citrate was subjected to velocity sedimentation, the major absorbing band sedimented more rapidly than a minor component.

It is of interest that the chloroplast-containing algae *Chlamydomonas reinhardi, Chlorella ellipsoidea,* and *Euglena gracilis* have major and minor bands of DNA, the concentration and density of which are very similar to the DNA of *Leishmania* (*7*). The trypanosomatid flagellate *Crithidia oncopelti* (*8*) contains a satellite band of the same density as the *Leishmania* minor DNA band. It would be of interest to know if these various minor bands of DNA, and also the DNA of the crab testis (*5*), form bands as rapidly in cesium chloride as does the DNA from *Leishmania* kinetoplasts.

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Ontogeny of Adventive Embryos of Wild Carrot

Abstract. *Somatic carrot cells in culture divide to produce undifferentiated preglobular proembryos which exhibit a wide variety of segmentation patterns. One or more globular proembryos, which exhibit normal histological zonation, may develop from single preglobular proembryos. Regeneration of normal embryos from cultured cells grown on media containing only minerals, sucrose, vitamins, and 2,4-dichlorophenoxyacetic acid suggests that the embryo sac may have less of a formative role than currently ascribed to it.*

The recent demonstration that cultured somatic cells of the wild carrot *Daucus carota*, when grown on simple defined media, will regenerate carrot embryos focuses attention on the forces which direct embryo development (*1, 2*). The view that embryo development is under considerable regulation by unique physical and chemical factors in the embryo sac (*3*) gained support from experimental studies which purported to show that coconut milk induced cultured cells to behave as zygotes (*4*). However, the occurrence of embryogenesis in the presence of coconut milk does not establish a causal relationship.

It is now clear that coconut milk is not required for the regeneration of embryos from cultured cells of the wild carrot and is, in fact, inhibitory to the development of carrot proembryos (*2*). It is also significant that in the only well-documented report of embryos from cultured cells of the domestic carrot (*5*), coconut milk was not used in the medium. This point needs to be emphasized since studies of the molecular basis for the regeneration of embryos from cultured cells will be hampered by the use of such complex substances as liquid endosperm.

We now present evidence that embryos which develop from wild carrot cells grown on a basal medium (*2*) containing only minerals, sucrose, vitamins, and 2,4-dichlorophenoxyacetic acid (2,4-D) undergo a series of developmental changes which may be more similar to embryogenesis in the ovule than previously reported, and that variations in the ontogeny of undifferentiated proembryos, caused by culture conditions, have little effect on subsequent embryo development.

We stated previously (*1*) that adventive embryos differed from seed embryos in that they usually did not have a suspensor-like component. This conclusion was erroneous, and attributable to the fact that most observations were made of embryos dissected from callus or embryos which developed in rotating liquid cultures—circumstances which led to the loss of the fragile suspensor (*6*). We have now studied numerous microtome sections of embryos embedded in callus in the original position where they formed, and it is evident that nearly always a distinct suspensor of variable size and shape is present (Fig. 1). In addition, we have grown groups of single cells and observed the entire developmental sequence from single cells to mature embryos. Evidently, the suspensor is nothing more than those cells of the proembryo (*7*) which are not incorporated into the embryo proper, and the size and shape of the preglobular proembryo varies, depending upon the number and orientation of mitoses which occur in random fashion prior to the establish-