Table 2. Effect of prior incubation upon LDH activity, as judged by the tetrazolium and DPN reduction methods. The indicated reaction components were incubated at 37°C for 8 minutes before addition of the remaining components. Control reactions were assayed with no prior incubation. Concentration of both DDT and heptachlor was 1×10^{-4} mole/liter.

Prior incubation mixture	Inhibition (%)	
	Tetrazolium	DPN
Enzyme + DPN + DDT	100	100
Enzyme + substrate + DDT	100	100
DPN + DDT	0	0
Substrate + DDT	0	0
Enzyme + DDT	100	100
Enzyme + DPN + heptachlor	100	100
Enzyme + substrate + heptachlor	100	100
DPN + heptachlor	0	0
Substrate + heptachlor	0	0
Enzyme + heptachlor	100	100

When the effect of chlorinated hydrocarbons upon lactate dehydrogenase was investigated, certain anomalies were noted, and I now report on their effect upon the data obtained from enzyme reactions in the presence of various pesticidal chemicals.

Lactate dehydrogenase activity was determined by the tetrazolium salt reduction method (*7*) and by the direct photometric measurement of DPN reduction at 340 mμ. Stock solutions of all pesticides (*8*) were prepared in isopropyl alcohol.

Except where otherwise noted, all reactions were carried out in a water bath at 37°C. After 20 minutes, the reactions were stopped by the addition of 0.5 ml of 0.5*N* HCl, and the optical density at 540 mμ was measured with a Bausch and Lomb Spectronic 20 colorimeter. In the control tubes the enzyme was added immediately before addition of the HCl.

The stimulation of LDH activity by the various chlorinated hydrocarbon compounds is observed when the tetrazolium reduction assay is used (Table 1). However, when the reaction is assayed by direct measurement of DPN reduction, a small inhibition of the reaction is noted.

Ceresan M inhibits the activity of LDH. The Ceresan M inhibition can be prevented by the addition of 2-mercaptoethanol to the reaction mixture. This, as well as the composition of the compound, suggests that the inhibition is due to the reaction of essential sulfhydryl groups with Ceresan M (*9*).

Although Table 1 indicates either a pronounced stimulation or a slight inhibition of LDH activity, depending upon assay method, Table 2 shows that prior incubation of either DDT or heptachlor with the enzyme results in severe inhibition. In addition, the inclusion of either substrate or DPN in the prior incubation mixture had no effect upon the observed inhibition.

These data indicate that the apparent stimulation of LDH activity by DDT and heptachlor when assayed by tetrazolium reduction is a result of interference with the mechanism of formazan production rather than a result of stimulation of enzyme activity. When the reaction is assayed by DPN reduction, there is no stimulation by either DDT or heptachlor. On the other hand, when DDT or heptachlor is incubated with LDH prior to addition of substrate, there is a complete inhibition of enzyme activity regardless of the method of assay. Thus DDT and heptachlor are capable of inhibiting the activity of LDH, although this is not apparent when the tetrazolium reduction assay is used without modification through prior incubation with an inhibitor.

The stimulation, attributed to an artifact, observed with the tetrazolium reduction method was further investigated by allowing the LDH reaction to proceed for 20 minutes in the presence of $1 \times 10^{-4}M$ DDT. The reactions were stopped as usual with HCl, and the DDT was removed by extraction with petroleum ether. The DDT in the extract was determined by means of gas chromatography (*10*) with an electron-capture detector. Only 60 percent of the added DDT could be recovered in the extracts. Control extractions were carried out by analysis of water solutions of DDT in the same manner. The controls showed 100 percent recovery of DDT. These data suggest the possibility that DDT forms a complex with a component of the reaction. Furthermore, when the concentration of iodophenylnitrophenyl tetrazolium chloride (INT) was doubled, only 40 percent of the added DDT could be recovered. While such data are not definitive, these results suggest the possibility of complex formation, perhaps with the INT.

The inhibition of various enzyme systems by chlorinated hydrocarbon compounds has been investigated frequently, and the results of such investigations have often been inconsistent and have led to considerable confusion. Therefore, the methods used for the determination of enzyme activity should be thoroughly evaluated before use in assay of enzymatic reactions involving chlorinated hydrocarbons.

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References and Notes

1. J. E. Sassida, *Science* **146**, 3647 (1964).
2. J. D. Judah, *Brit. J. Pharmacol.* **4**, 120 (1949).
3. B. Sactor, *J. Econ. Entomol.* **43**, 832 (1950).
4. C. D. Johnston, *Arch. Biochem. Biophys.* **31**, 375 (1951).
5. M. M. Nachlas, S. I. Margulies, A. M. Seligman, *J. Biol. Chem.* **235**, 2 (1960).
6. R. D. O'Brien and F. Matsumura, *Science* **146**, 657 (1964).
7. M. M. Nachlas, S. I. Margulies, J. D. Goldberg, A. M. Seligman, *Anal. Chem.* **1**, 317 (1960).
8. Ceresan M, *N*-(ethylmercuri)-*p*-toluenesulfonanilide, Thiram, bis(dimethylthiocarbamoyl) disulfide, DDT, DDE, Heptachlor, aldrin, dieldrin, and endrin are all complex chlorinated hydrocarbons. For detailed structures of these compounds see *The Merck Index* (Merck and Company, Inc., Rahway, N. J., 1960).
9. D. R. Kominz, *Science* **149**, 3690 (1965).
10. J. Burke and W. Holswade, *J. Off. Agr. Chem.* **47**, 5 (1964).

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Nuclei from Rat Liver: Isolation Method That Combines Purity with High Yield

Abstract. *A procedure is described that gives almost quantitative separation of clean nuclei from a homogenate of rat liver. It is a modification of methods that use concentrated sucrose solutions rather than citric acid or detergents and, therefore, permits further quantitative fractionation of cytoplasmic components.*

A recent review (*1*) has compared various methods for the preparation of nuclei from rat liver, which has usually been done by a series of differential centrifugations in isotonic sucrose. Approximately 100 percent of nuclei in the homogenate were recovered, but preparations also contained 12 to 14 percent of cellular RNA (*2*). Introduction of more concentrated sucrose solutions (2.2*M*) by Chauveau *et al.* (*3*) permitted recovery

of 45 to 55 percent of the nuclei after only one centrifugation; nuclear RNA represented only 6 to 7 percent of total cellular RNA and was the smallest proportion to be reported up to that time. Electron micrographs showed these nuclei to be intact. Decrease in RNA content was attributed to decreased contamination with cytoplasmic RNA (presumably the endoplasmic reticulum, with its attached ribosomes, which is continuous with the outer nuclear membrane). Removal of the endoplasmic reticulum is thought to be achieved through a shearing effect. The density of Chauveau's sucrose solution is intermediate between the higher density of nuclei and the lower density of endoplasmic reticulum. Thus, when centrifugal force is applied, nuclei tend to move down through the solution while endoplasmic reticulum moves upward, and attached fragments of the endoplasmic reticulum are stripped from the nuclei, leaving the outer nuclear membrane relatively intact.

The Chauveau method can result in some degree of cytoplasmic contamination, however, since the sample is initially distributed evenly throughout the tube. Those particles with a density equal to or higher than that of nuclei (that is, free ribosomes) and which are initially near the bottom of the tube will sediment into the pellet with nuclei. Furthermore, nuclei initially near the bottom of the tube will sediment so rapidly that the shearing action will be minimal.

In a study of the Chauveau procedure by Maggio, Siekevitz, and Palade (*4*), a slight but definite contamination was shown. This contamination could be eliminated by layering a homogenate in 0.88*M* sucrose medium over 2.2*M* sucrose medium. The yield, however, was reduced to 25 to 30 percent of the total nuclei because of an accumulation of endoplasmic reticulum and mitochondria at the interface, which then trapped many nuclei.

In our study, by raising the sucrose concentration of the homogenate to a density just sufficient to float the endoplasmic reticulum and mitochondria, thereby eliminating their accumulation at the interface, and by raising the sucrose concentration of the bottom layer to 2.3*M*, we were able to recover more than 90 percent of the nuclei with very little cytoplasmic contamination, as evidenced by electron microscopy, and an even lower proportion of cellular RNA in the nuclear fraction (4 to 5 percent). Our procedure, furthermore, avoids the high dilution of homogenate used in both the original Chauveau method and the modification of Maggio *et al.* (*4*). This higher concentration of homogenate was advantageous in further fractionation of the cytoplasmic components, that is, free and bound ribosomes (*5*).

Table 1. Amounts of DNA and RNA in rat liver homogenate and in the nuclei. Values given are from 1.0 ml of homogenate (33 percent by weight and volume) and in the nuclear pellet isolated from it (details as described in the text). Mean values given in footnotes are ± standard error of the mean.

Rat No.	DNA in			RNA in			RNA/DNA in homogenate ‡	RNA/DNA in nuclear pellet §
	Homogenate (mg)	Nuclear pellet (mg)	Nuclear pellet (%)*	Homogenate (mg)	Nuclear pellet (mg)	Nuclear pellet (%) †		
				Animals fasted				
1	1.230	1.109	90.1	2.62	0.118	4.50	2.13	0.106
2	1.260	0.965	76.6	3.01	.108	3.58	2.39	.111
3	1.440	1.330	92.4	3.07	.143	4.65	2.13	.107
				Animals fed				
4	0.815	0.770	94.4	2.30	0.095	4.12	2.82	0.124
5	.890	.857	96.3	2.40	.112	4.65	2.70	.130
6	.880	.857	97.4	2.60	.107	4.13	2.95	.125

* Mean for fasted and fed animals, 91 ± 3. † Mean for fed animals, 4.3 ± 0.2. ‡ Mean for fasted animals, 2.22 ± 0.09; for fed animals, 2.82 ± 0.07. § Mean for fasted animals, 0.108 ± 0.002; for fed animals, 0.126 ± 0.002.

All experiments were performed with male albino rats (weight, 150 to 200 g) of the Holtzman strain (*6*). They were housed in a room with controlled light from 9:00 a.m. to 9:00 p.m. and were fed a 60-percent protein diet (*7*) only during the dark hours every other night (that is, they were fasted for 36 hours and fed for 12 hours in each 48-hour period) (*8*). This feeding regimen provides fed and fasted animals with known periods. One group of rats was killed at 9:00 a.m. after feeding, another group at 9:00 p.m. after a 36-hour fast. The livers were removed quickly and chilled immediately in several volumes of ice-cold 0.25*M* sucrose in TKM (0.05*M* tris-HCl, *p*H 7.5, at 20°C; 0.025*M* KCl; and 0.005*M* $MgCl_2$). All subsequent operations were performed at temperatures near 0°C. Livers were blotted, weighed, and minced with scissors in two volumes of ice-cold 0.25*M* sucrose in TKM. They were homogenized in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (clearance 0.025 cm) with 10 to 15 strokes at 1700 rev/min. The homogenate was filtered through four layers of cheese cloth. Subsequent fractionation of the homogenate is described below. RNA was determined according to Fleck and Munro (*9*), with the extinction coefficient, $E_{1\ cm}^{1\%}$, equal to 312 [32 μg/ml = 1.000 optical density unit, according to Munro and Fleck (*10*)] rather than with $E_{1\ cm}^{1\%}$ equal to 213 (corresponding to 47 μg/ml = 1.000 OD) as given originally by Fleck and Munro (*9*). DNA was determined according to the Ceriotti procedure (*11*) with 2.5*N* instead of concentrated HCl (*12*) and with heating for 20 instead of 10 minutes. Calf thymus DNA (*13*) was used as a standard. Nuclear pellets were fixed *in situ*, that is, at the bottom of the tube, for 35 minutes at 0°C and then fixed in 1 percent OsO_4 buffered with PO_4 (*14*), dehydrated in ethanol and propylene oxide, and embedded in a mixture of Araldite and Epon according to standard procedures. Sections were stained with lead citrate and viewed in a Hitachi electron microscope (HU-11B-2).

In our procedure 1.0 ml of homogenate, prepared as described above, was pipetted into a polyallomer tube that fit the SW 39 Spinco rotor; 2.0 ml of 2.3*M* sucrose in TKM was then added by means of a syringe and 13-gauge needle (rather than with a pipette, because of the high viscosity of the solution) and thoroughly mixed with the 0.25*M* sucrose homogenate by inversion. Sucrose concentration of the homogenate was thereby raised to approximately 1.62*M*, the density of which is just sufficient to float mitochondria and rough endoplasmic reticulum. The mixture was then underlaid by 1.0 ml 2.3*M* sucrose in TKM with a syringe and 13-gauge needle: tip of the needle was placed

at the bottom of the tube and the heavy sucrose solution introduced, forcing the lighter homogenate upward. After centrifugation for 30 minutes at 39,000 rev/min in a Spinco SW 39 rotor ($124{,}000g_{av}$) at 0° to 4°C, the supernatant was poured off. Material adhering to the wall of the tube was removed with a spatula and added to the supernatant; the tube wall was then wiped dry with tissue paper wrapped around a spatula. The white nuclear pellet was taken up in TKM buffer and analyzed for RNA and DNA.

Data in Table 1 show that 91 ± 3 percent of DNA in the filtered homogenate was recovered in the nuclear pellet. According to Schneider and Kuff (*15*), 98.5 percent of DNA of rat liver cells is located in the nucleus and 1.5 percent in the mitochondria. Therefore, DNA in the pellet actually represents slightly more than 91 percent of nuclear DNA. Only 4.3 percent of the total RNA in the filtered homogenate was recovered in the nuclear pellet. If one assumes that no nuclear RNA was extracted during isolation and corrects for 100 percent recovery of nuclei, nuclear RNA would amount to only 4.7 percent of the total cellular RNA. Maggio *et al.* (*4*) showed that no significant amount of nuclear RNA can be extracted by buffers of low ionic strength (0.1*M* phosphate, *p*H 7.1). Since the TKM buffer we used was of even lower ionic strength, extraction of nuclear RNA by the buffer was unlikely. In fact, replacing TKM by 0.003*M* $CaCl_2$ in our procedure gave the same quantitative relation of nuclear to homogenate RNA. The 4.7 ± 0.2 percent nuclear RNA we report here is slightly lower than the 6 to 7 percent reported by Chauveau, which, however, was calculated from a nuclear yield of only 45 to 55 percent (*2*). Ratios of RNA to DNA in animals fasted for 36 hours were 2.22 ± 0.09 in the total homogenate and 0.108 ± 0.002 in the nuclear pellet; the ratios for fed rats were significantly higher for both homogenate and nuclei. All values are in good agreement with compiled data in the literature (*1, 16*).

Table 2. Effect on RNA and DNA content of nuclei after washing them with detergents. Nuclei isolated from 1.0 ml homogenate were resuspended in 2.0 ml of 0.25*M* sucrose in TKM. For each sample, 1.8 ml of this pooled solution was mixed with 0.2 ml of buffer or detergent as indicated. Samples were centrifuged for 5 minutes at 800*g*, and pellets were analyzed for RNA and DNA.

Nuclei washed with	DNA (mg)	DNA (%)	RNA (mg)	RNA (%)
TKM buffer	0.953	100	0.111	100
Triton X-100 in TKM:				
5% (wt/vol)	1.018	107	.106	96
10% (wt/vol)	0.925	97	.104	93
20% (wt/vol)	.994	99	.105	95
Deoxycholate and Tween 40*	.907	95	.103	93

* See (*8*).

Although most of the nuclei appeared intact when viewed with a phase microscope, the electron micrographs showed that approximately 50 percent of them were damaged. This partial damage did not seem to cause any significant loss of nuclear RNA, as shown by experiments with radioactive RNA precursors (*17*). Thirty minutes after injection of labeled orotic acid in vivo, when most of the radioactivity is localized in nuclear RNA, it was found that 85 to 90 percent of labeled RNA was recovered in nuclei isolated by our method. This is good evidence that the method does not lead to loss of nuclear RNA.

Electron micrographs (Fig. 1A) show that the isolation procedure does not remove the outer nuclear membrane with its attached ribosomes; the latter can be considered as a form of cytoplasmic contamination. Penman (*18*) recently demonstrated that the outer nuclear membrane of HeLa cell nuclei can be completely removed with a mixture of deoxycholate and Tween 40. In our hands, this procedure caused a complete fragmentation of rat liver nuclei as seen with the electron microscope, although only small amounts of DNA and RNA were lost (Table 2). Triton X-100, however,

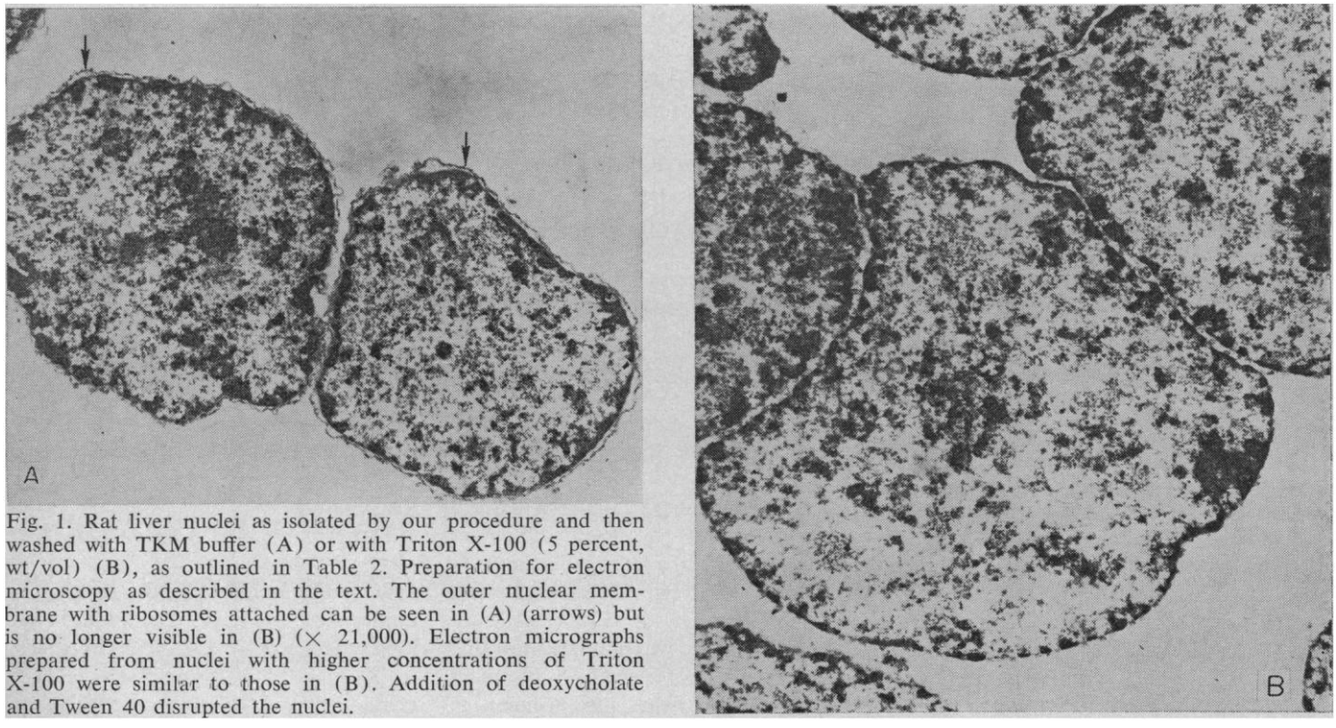

Fig. 1. Rat liver nuclei as isolated by our procedure and then washed with TKM buffer (A) or with Triton X-100 (5 percent, wt/vol) (B), as outlined in Table 2. Preparation for electron microscopy as described in the text. The outer nuclear membrane with ribosomes attached can be seen in (A) (arrows) but is no longer visible in (B) (× 21,000). Electron micrographs prepared from nuclei with higher concentrations of Triton X-100 were similar to those in (B). Addition of deoxycholate and Tween 40 disrupted the nuclei.

which had previously been used in 0.5-percent concentration for isolation of nuclei (*19*), was, in concentrations of 0.5 to 2 percent, effective in removing the outer nuclear membrane without further disruption of the nuclei (Fig. 1B). There was only a small (4 to 7 percent) loss of nuclear RNA and virtually no loss of DNA (Table 2). It is not clear whether the loss of RNA after treatment with detergent can be attributed solely to removal of the outer nuclear membrane and its attached ribosomes. It is conceivable that some of the nuclear RNA is made soluble by the detergent and no longer sediments with the nuclei. Most of the nuclear RNA, however, is bound by a mechanism which is clearly resistant to the tested concentrations of Triton X-100. This is distinctly different from the situation in the cytoplasm, where 60 percent of the RNA (*5*) is bound to membranes as ribosomes and can be released by 2 percent Triton solutions.

Our method avoids use of citric acid completely and avoids use of detergent until the nuclei have been separated from the cytoplasm and thus permits further fractionation of the cytoplasm, as will be shown elsewhere (*5*). Removal of traces of the endoplasmic reticulum from nuclei by means of Triton X-100 can be carried out optionally, and further studies on the advantages or disadvantages in each specific application may be necessary.

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References and Notes

1. D. B. Roodyn, *Biochem. Soc. Symp.* **23**, 20 (1963).
2. W. C. Schneider, *J. Biol. Chem.* **176**, 259 (1948).
3. J. Chauveau, Y. Moule, C. Rouiller, *Exp. Cell Res.* **11**, 317 (1956).
4. R. Maggio, P. Siekevitz, G. E. Palade, *J. Cell Biol.* **18**, 267 (1963).
5. G. Blobel and V. R. Potter, in preparation.
6. Holtzman Co., Madison, Wisconsin.
7. General Biochemicals, Chagrin Falls, Ohio.
8. V. R. Potter, R. A. Gebert, H. C. Pitot, *Advance. Enzymol.* **4**, 247 (1966).
9. A. Fleck and H. N. Munro, *Biochim. Biophys. Acta* **55**, 571 (1962).
10. H. N. Munro and A. Fleck, *Analyst* **91**, 78 (1966).
11. G. Ceriotti, *J. Biol. Chem.* **198**, 297 (1952).
12. K. Keck, *Arch. Biochem. Biophys.* **63**, 446 (1956).
13. Mann Research Laboratories, New York, Lot K 1742 with P, 9.04 percent and N, 14.56 percent.
14. G. Millonig, *J. Appl. Phys.* **32**, 1637 (1961).
15. W. C. Schneider and E. L. Kuff, *Proc. Nat. Acad. Sci. U.S.* **54**, 1650 (1965).
16. I. Leslie, in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, Eds. (Academic Press, New York, 1955), vol. 2, p. 1.
17. R. A. Gebert, G. Blobel, V. R. Potter, in preparation.
18. S. Penman, *J. Mol. Biol.* **17**, 117 (1966).
19. W. C. Hymer and E. L. Kuff, *J. Histochem.* **12**, 359 (1964).
20. Triton X-100 was a gift from Rohm and Haas Co., Philadelphia; Tween 40 was purchased from Nutritional Biochemicals Corp., Cleveland; and sodium deoxycholate from Matheson, Coleman and Bell, Cincinnati.
21. Supported by departmental grant CA-07125 from PHS. We thank Nancy Gutensohn and G. Pridmore for preparation of electron micrographs. G.B. is a PHS postdoctoral fellow.

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Interaction of the Water-Soluble Carcinogen 4-Nitroquinoline N-Oxide with DNA

Abstract. *Thin-layer chromatography revealed that 4-nitroquinoline* N*-oxide interacts with native DNA, but not with denatured DNA's or the following macromolecules and nucleic acid derivatives: soluble RNA from* Escherichia coli *and from yeast, polyguanylate-cytidylate, and precursors of DNA and RNA. Polydeoxyadenylate-thymidylate, polyadenylate, and histone interacted to a lesser degree than native DNA's did. That magnesium ion, sodium ion, and proflavine did not interfere with interaction of carcinogen and DNA suggests that this interaction is not an intercalation. Interaction of 4-nitroquinoline* N*-oxide with DNA may be related to its carcinogenicity.*

Proximate derivatives of such carcinogens as the amino-azo dyes, aminofluorenes (and perhaps of the naphthylamines and polycyclic benzenoid hydrocarbons) are usually more polar than the parent carcinogens (*1*). This increases interest in the polar, water-soluble carcinogen 4-nitroquinoline *N*-oxide (NQO) (Fig. 1a) as a model compound for investigating the carcinogenic process (*2*). It has recently been suggested that a reduced form of NQO, 4-hydroxyaminoquinoline *N*-oxide, may be the proximate form of this carcinogen (*3*).

The manifold carcinogenicity of NQO rivals that of the most potent polycyclic hydrocarbons (*4*). Its carcinogenic properties are expressed after its administration by skin painting, ingestion, or various routes of injection (*5*). It is carcinostatic and also mutagenic for bacteria and yeast (*6*). In Chang liver cells and in the protozoan *Tetrahymena pyriformis*, NQO elicits visible intranuclear inclusion bodies (*7*), sharing with mitomycin C an ability to induce nucleolar caps (*8*). It also bleaches *Euglena*, induces chromosomal aberrations in Yoshida sarcoma cells, and diminishes uptake of ^{32}P into nucleic acid fractions of the Ehrlich ascites carcinoma (*9*). We therefore studied the interaction of NQO with total extracted *Euglena* DNA as well as with DNA from other sources. The choice of *Euglena* DNA might, moreover, permit insight into the bleaching process because of its relation to the autonomy of organelles, such as the chloroplast, whose DNA's differ from nuclear DNA's (*10*). Our results show that NQO does interact with native DNA from *Euglena* and other sources.

The spectrum of NQO (Fig. 1b) has a sharp peak at 250 mμ and a broad peak at 370 mμ. The overlapping spectra of NQO and DNA at 250 mμ preclude accurate spectrophotometric analysis at this wavelength. Use of the peak at 370 mμ as an index of binding is not a conclusive means of demonstrating interaction between NQO and either DNA or RNA (*11*). To surmount the problem posed by overlapping spectra, we used thin-layer chromatography (TLC): Eastman Chromagrams with distilled water or a mix-

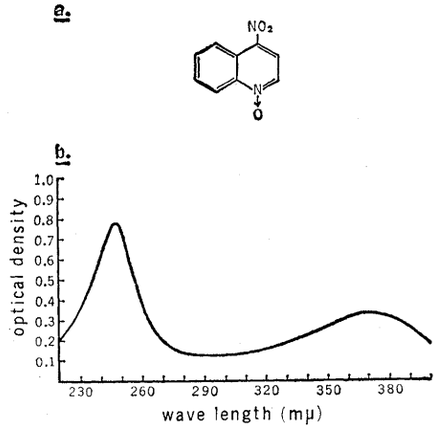

Fig. 1. (a) Structure of 4-nitroquinoline *N*-oxide, molecular weight 191.1. (b) Absorption spectrum of NQO in 0.1 times SSC buffer, *p*H 7.2, 8.1 μg/ml.