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Inhibition of [6-^{14}]Orotic Acid Incorporation into the Cytosine Moiety of the Ribonucleic Acid of Rat Liver Cytoplasmic Ribosomes after Phenobarbital Administration

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SUMMARY

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Administration of phenobarbital in drinking water (1 g/liter) to rats produces a disproportionate incorporation of [6-^{14}C]orotic acid into the pyrimidine nucleotides of the RNA of the isolated cytoplasmic ribosomes and also tRNA. The specific activity of uridylic acid is slightly decreased, whereas the specific activity of cytidylic acid is markedly lowered. After correction for the increased rRNA content in hypertrophic liver following the administration of phenobarbital, the specific activity of uridylic acid is only slightly increased, although the decrease of the value for cytidylic acid is still apparent. The decreased incorporation of labeled orotic acid is also evident in the cytosine components of the nucletoide pool. The observed differences in the incorporation of [6-^{14}C]orotic acid into both pyrimidine nucleotides of rRNA depend on the length of application of the drug: the largest differences are observed between the 6th and 20th days of phenobarbital administration. A slight convergence of the values occurs during the subsequent phase of the application.

INTRODUCTION

A number of recent observations have indicated that phenobarbital affects the synthesis and degradation of RNA in rat liver. Examination of the half-life of isolated liver ribosomes (1) and of ribosomal RNA (2) showed that the drug not only activates the synthesis of ribosomal RNA but also prolongs its half-life in the cell. It appears that the increased half-life of cytoplasmic ribosomes is associated with the inhibition of their degradation during the period of hypertrophy of the liver cell after repeated administration of phenobarbital. Furthermore, the synthesis of nonribosomal RNA of microsomal membranes is inhibited (3). At the same time it was observed that the administration of the drug decreases the activity of liver ribonucleases, not only in the microsomal (4–7) but also in other subcellular fractions of the liver (7) and isolated ribosomes (8).

Since protein synthesis is functionally connected with the synthesis of all types of RNA, a study of RNA metabolism in the liver following application of phenobarbital may contribute to the understanding of the molecular mechanism of induction of drug-metabolizing enzymes (9, 10) and liver hypertrophy. In the present communication disproportionate incorporation of labeled orotic acid into uridylic and cytidylic acids of ribosomal and transfer RNA following the administration of phenobarbital to rats is reported.

METHODS

Male rats (Wistar, Lysolaje strain), weighing 150 g at the beginning of the experiment, were given phenobarbital (sodium salt, Merck) in drinking water (1 g/liter). [6-^{14}C]Orotic acid (Amersham; specific activity, 60.8 mCi/mmole) was administered intraperitoneally in 0.9% NaCl. Unless otherwise stated, rats were given 10 μCi of [6-^{14}C]orotic acid per kilogram of body weight. Animals were fasted for 12 hr before decapitation. Each experimental group contained three rats.

Total liver RNA was determined according to a modified procedure of Ogur and Rosen (11) as previously described (12). Aliquots of the hydrolysate were neutralized by potassium hydroxide and the specific activity was determined. The incorporation into RNA of whole liver was calculated from these values.

The liver ribosomes were isolated according to Munro *et al.* (13). For the isolation of tRNA, the postmitochondrial fraction was centrifuged at 150,000 $\times$ *g* for 2 hr and the supernatant solution was used for the isolation of tRNA by the phenol method (14). Sodium deoxycholate then was added to the postmitochondrial supernatant fraction. The ribosomes were sedimented by centrifugation at 150,000 $\times$ *g* for 1 hr. The ribosomal pellet was washed with sucrose-free buffer and centrifuged as described above. The ribosomal pellets and isolated tRNA were resuspended and hydrolyzed in sealed ampoules with 1 N HCl at 100° for 1 hr (15). The ribosomal RNA hydrolysate was centrifuged to remove precipitated proteins and then applied to Whatman No. 3 chromatographic paper. The pyrimidine nucleotides and the purine bases were separated in a system containing 2-propanol–HCl–H_2O (170:41:39) (16). 3′-CMP and 3′-UMP were detected under ultraviolet light; the absorbing zones were cut out and eluted by a continuous flow of water, and their absorbance was measured in 0.01 N HCl. To calculate the concentration of 3′-CMP, a molar absorptivity value of $\lambda_{278} = 13.00$, and for 3′-UMP $\lambda_{262} = 9.90$, was used. The radioactivity was measured in a neutral aliquot of the hydrolysate and is expressed in counts per minute per micromole of pyrimidine nucleotide.

To determine the pool of free nucleotides, the excised liver was immediately homogenized with 5 volumes of cool 5% trichloracetic acid. After the centrifugation the sediment was extracted once more with 2.5 volumes of 5% trichloroacetic acid. The extracts were pooled, and trichloracetic acid was removed by repeated extraction with ethyl ether until the solution was neutral. The absorbance of the liver extracts at 260 nm and their radioactivity was measured. From these values the radioactivity per gram of liver was calculated. An aliquot of the extract was taken to dryness and hydrolyzed in 70% $HClO_4$ for 1 hr at 100°. After neutralization with potassium hydroxide the specific activity of the total pool of free nucleotides in the supernatant fraction was determined. Aliquots of the hydrolysate were applied to Whatman No. 3 chromatographic paper, and the pyrimidine bases were separated in isobutyric acid–water–concentrated ammonia (66:33:1.5). The zones corresponding to uracil and cytosine were eluted with a continuous flow of water, and the ratio of radioactivity of cytosine to that of uracil was determined in the eluate. The radioactivity was measured with a Packard spectrometer in a scintillator containing 2,5-diphenyloxazole, 4 g; 1,4-bis-[2-(5-phenyloxazolyl)]benzene, 100 mg; and naphthalene, 120 g, per liter of dioxane.

RESULTS

After 7 days of phenobarbital treatment the total RNA content per unit of weight was practically identical with the content of total liver RNA of the control group (6.9 mg for control rat liver and 6.6 mg/g of liver in experimental animals). Total liver RNA after phenobarbital administration thus increased in proportion to the weight of the organ.

Analysis of the ratio of specific activities or uridylic and cytidylic acids showed an uneven distribution of radioactivity between these pyrimidine components of rRNA after the administration of phenobarbital. The specific activities of uridylic acid were slightly lower in the experimental group

than in the control group (Fig. 1A). When these values are weighted for liver hypertrophy (i.e., for the increased amount of ribosomes), however, the experimental group exhibits somewhat higher specific activities for uridylic acid (Fig. 1B).

The specific activities of cytidylic acid of rRNA were markedly lower after the administration of phenobarbital at all time intervals investigated (Fig. 2A), even after allowing for changes in liver weight (Fig. 2B). The ratio of the specific activities of uridylic and cytidylic acids is shown in Fig. 3. In the experimental group this ratio was higher at all intervals investigated after the administration of [6-^{14}C]orotic acid. After 60 hr this ratio in the control group equaled 1.0, compared to the experimental group ratio of 1.8.

Decreased incorporation of labeled orotic acid after the administration of phenobarbital could also be observed with liver tRNA. The relative decrease of specific activity of cytidylic acid compared to uridylic acid was practically the same as in rRNA (Table 1).

The investigation of incorporation of labeled orotic acid into the RNA of liver cytoplasmic ribosomes as a function of treatment with phenobarbital showed that the specific activity of UMP increased slightly in the experimental group during the first days of treatment with the drug; later it slowly decreased and remained at a depressed level during the entire period of chronic treatment with phenobarbital (Fig. 4). When phenobarbital administration was discontinued after 7 days, the specific activity of uridylic acid returned to the control levels 4 days later. The specific activities of

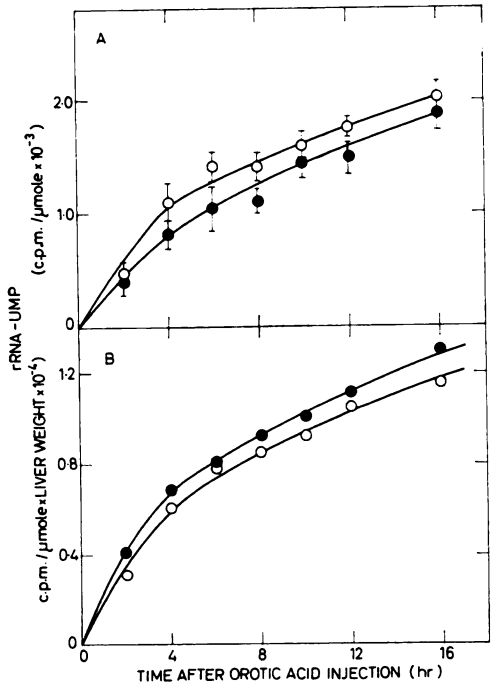

FIG. 1. *Incorporation of [6-^{14}C]orotic acid into uridylic acid of rRNA*

A. Specific activity of UMP. B. Specific activity of UMP × liver weight. O——O, controls; ●——●, after 7 days of phenobarbital treatment. The brackets indicate the standard errors for six determinations.

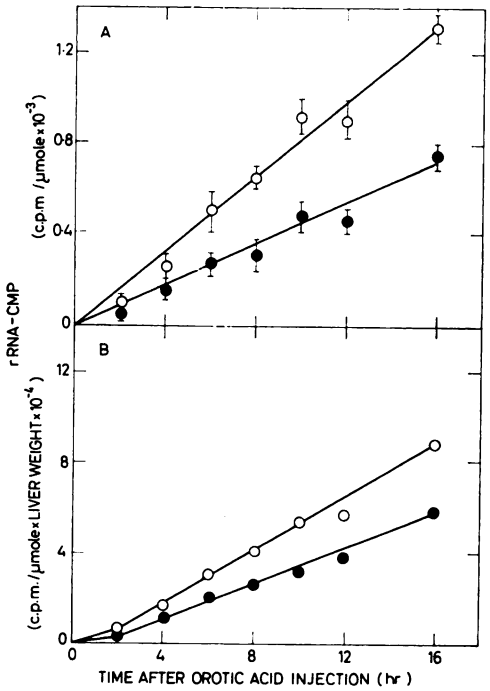

FIG. 2. *Incorporation of [6-^{14}C]orotic acid into cytidylic acid of rRNA*

A. Specific activity of CMP. B. Specific activity of CMP × liver weight. O——O, controls; ●——●, after 7 days of phenobarbital treatment. The average liver weight of controls was 6.0 g; that of phenobarbital-treated animals was 8.4 g. The brackets indicate the standard errors for six determinations.

cytidylic acid decreased from the beginning of phenobarbital administration and were lowest after the sixth day; starting from the 20th day a slow increase was observed. The specific activity of cytidylic acid returned to control levels 6 days after discontinuation of the drug.

The ratio of specific activities of uridylic and cytidylic acids paralleled the extent of hypertrophy of the liver after administration of phenobarbital, and also the regression of liver weight after treatment with the drug was discontinued (Fig. 5).

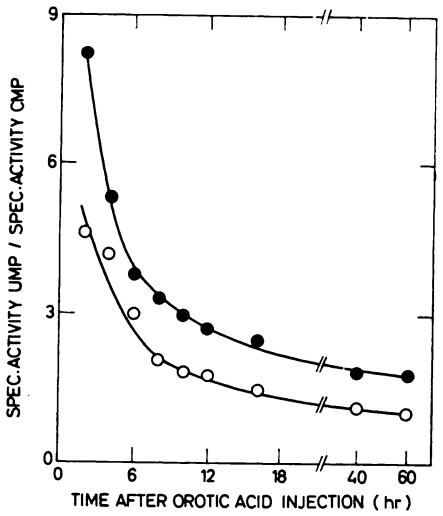

FIG. 3. *Ratio of specific activities of pyrimidine nucleotides of rRNA*

○——○, controls; ●——●, after 7 days of phenobarbital treatment.

Determination of the total radioactivity of the acid liver extract indicated that the radioactivity values in the experimental group approached those for the control group. Similarly, there were no differences in the total absorbance at 260 nm of acidic extracts per gram of liver. Small differences were found in the specific activities of the hydrolyzed free nucleotide pool (Table 2). The decrease of specific radioactivity in the free nucleotide pool decreased with time in a semilogarithmic fashion. In the treated group a decreased rate of decline in the specific activity of the pool was observed (Fig. 6).

In a hydrolysate of the free nucleotide pool the majority of radioactivity was present as uracil. In the phenobarbital-treated group, the incorporation of labeled orotic acid into the cytosine of the free nucleotide pool was markedly decreased at all time intervals examined after the administration of labeled precursors (Fig. 7).

DISCUSSION

The administration of phenobarbital induces the synthesis of microsomal enzymes of drug metabolism (8, 10). At the same time the liver weight increases in parallel with the proliferation of smooth endoplasmic reticulum (17). The use of histochemical (18) and biochemical (19) methods has

TABLE 1

Incorporation of [6-^{14}C]orotic acid into pyrimidine nucleotides of cytoplasmic RNAs of liver

The animals were killed 20 hr after the injection of labeled orotic acid. The average liver weight was 5.6 ± 0.6 g for controls and 8.3 ± 0.9 g for rats after 7 days of phenobarbital treatment. The values for rRNA represent the means ± standard errors of 10 determinations, and for tRNA, the means of six determinations.

RNA species	3′-UMP			3′-CMP			Molar ratio, CMP:UMP[a]	
	Controls	Phenobarbital-treated	Per cent of controls	Controls	Phenobarbital-treated	Per cent of controls	Controls	Phenobarbital-treated
	cpm/μmole			*cpm/μmole*				
rRNA	2450 ± 350	1860 ± 240	76	1650 ± 210	640 ± 90	39	1.75	1.70
tRNA	2500	1940	77	2100	770	37	1.50	1.44

[a] These values represent the micromolar ratio present in the HCl hydrolysate of RNAs.

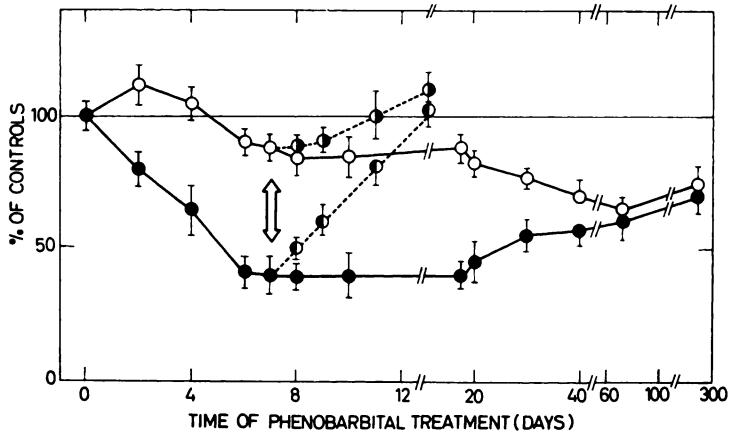

FIG. 4. *Incorporation of [6-^{14}C]orotic acid into pyrimidine nucleotides of isolated cytoplasmic ribosomes during chronic treatment with phenobarbital*

○——○ and ●——●, specific activities of UMP and CMP, respectively; ◑——◑ and ◐——◐, specific activities of UMP and CMP after discontinuation of phenobarbital treatment. The rats were killed 20 hr after the injection of labeled orotic acid. The arrow indicates the interruption of phenobarbital treatment. The brackets indicate the standard errors for six determinations.

shown that the increase in cytoplasmic volume of the liver cell is predominantly responsible for the increase of liver weight after the administration of phenobarbital. The DNA content decreases in proportion to the increasing liver weight, while the RNA content per unit weight is the same as in the control group. The total RNA content of the liver after administration of phenobarbital is thus proportional to the

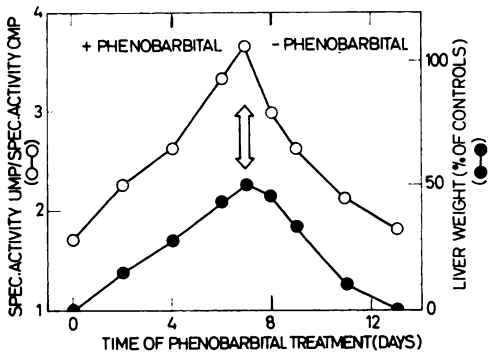

FIG. 5. *Ratio of specific activities of UMP and CMP and liver weight changes after repeated administration of phenobarbital*

○——○, UMP:CMP (left ordinate); ●——●, liver weight (right ordinate). The rats were killed 20 hr after the injection of labeled orotic acid. The arrow indicates the discontinuation of phenobarbital treatment.

liver hypertrophy. This finding is important for the evaluation of changes in the specific activities of isolated cytoplasmic ribosomes after administration of phenobarbital. Since more than 90 % of the liver RNA is ribosomal (20), the increase in total RNA in the liver cell reflects a proportional increase in the number of ribosomes. The specific activity of rRNA after the administration of phenobarbital will be affected by the dilution of the labeled ribosomes by unlabeled ribosomes, which in the experimental group were present in a greater number in the cell. Since the half-life of ribosomes in the livers of the weight category employed is 4–5 days (1), it cannot be assumed that appreciable degradation of labeled ribosomes and their RNAs could take place durig the time intervals investigated. After correction of the values for changes in liver weight, it becomes obvious that the specific activity of uridylic acid was only slightly higher in the phenobarbital-treated group than in the control group. In contrast, correction for liver weight does not eliminate the differences in the specific activities of cytidylic acid. Since uridine nucleotides are predominantly labeled in rRNA during short-term contact with [6-^{14}C]orotic acid, the decrease of specific activity of total rRNA cannot be ob-

TABLE 2

Radioactivity of acid-soluble extracts and of pool of soluble nucleotides of liver after injection of [6-^{14}C]orotic acid

Phenobarbital-treated animals received the drug for 7 days. The values are the means and standard errors of six determinations.

Time after labeled orotic acid	Treatment	Radioactivity of liver extracts	Specific activity of $HClO_4$ hydrolysates
hr		*(cpm/g liver) × 10^{-3}*	*cpm/A_{260}*
1	Control	273 ± 48	2100 ± 350
	Phenobarbital	265 ± 55	1940 ± 340
2	Control	255 ± 40	1950 ± 290
	Phenobarbital	225 ± 44	1860 ± 305
4	Control	240 ± 53	1710 ± 270
	Phenobarbital	210 ± 44	1520 ± 260
8	Control	168 ± 44	1250 ± 205
	Phenobarbital	167 ± 36	1330 ± 180

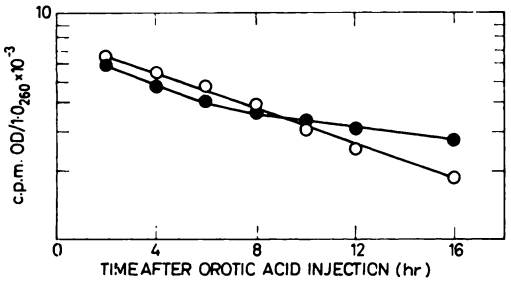

FIG. 6. *Decline of specific activities of pool of soluble liver nucleotides after injection of [6-^{14}C]orotic acid (25 μCi/kg of body weight)*

○——○, controls; ●——●, after 7 days of phenobarbital treatment. The values are the means of five determinations.

served at early intervals after administration of the labeled precursor. We have not observed any marked differences in the molar ratio of both pyrimidine nucleotides in rRNA or tRNA after repeated administration of the drug.

Our subsequent investigations were focused on the potential effect of phenobarbital on the composition and metabolism of the pyrimidine pool. Quantitative analysis of the total acid liver extract showed no significant difference between the experimental and the control group in the quantity per unit of tissue weight or in total radioactivity. This finding indicates that the changes described above are not caused by a difference in the penetration of the labeled precursor into the liver cells. Since we did not find any essential differences between the specific activities of uridine nucleotides of rRNA, it is unlikely that the changes in CTP synthesis could result from differences in UTP level.

The synthesis of the pyrimidine nucleotides is governed by a number of metabolic controls, including feedback inhibition and feedback repression by the final products of metabolism. Since a slowdown in the decrease of the nucleotide pool specific radioactivity could be observed in the group of experimental animals, it is possible that the decrease in cytidine nucleotide synthesis is caused by the inhibitory effect of one of the components of the nucleotide pool whose concentration had changed after the administration of phenobarbital. Decreased degradation of uracil after the administration of phenobarbital has been observed in mouse liver (21).

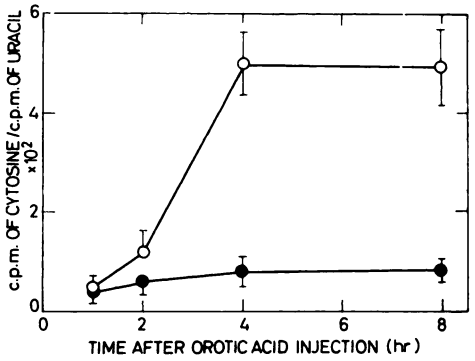

FIG. 7. *Incorporation of [6-^{14}C]orotic acid into the cytosine moiety of hydrolyzed acid-soluble pool of liver*

○——○, controls; ●——●, after 7 days of phenobarbital treatment. The brackets indicate the standard errors for eight determinations.

It remains to be shown how the depression of cytidine nucleotide synthesis is connected with enzyme induction following the administration of phenobarbital. The time course of the decrease in specific activities of cytidylic acid in the RNA of cytoplasmic ribosomes points to a discrete relation with liver hypertrophy. The specific activities of cytidylic acid are lowest after the sixth day, i.e., during the so-called "steady state," when additional administration of the drug does not increase liver weight or the activity of the drug-metabolizing enzymes (22–24). After the administration of phenobarbital has been interrupted, the specific activities of cytidylic acid return to control values in a few days, paralleling the regression of liver weight. After long-term administration of the drug the differences in the specific activities of the two pyrimidine nucleotides of rRNA disappear. When these activities are corrected for the higher content of ribosomes in the cell, the differences in the specific activity of rRNA also disappear.

We may thus conclude that phenobarbital, in addition to its effect on the synthesis and degradation of ribosomes and their component ribonucleic acids, also affects the synthesis *de novo* of the low molecular weight pyrimidine precursors. The biochemical mechanism of this phenomenon is being studied further.

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REFERENCES

1. J. Seifert, J. Vácha, and H. Remmer, *Proc. Eur. Soc. Study of Drug Toxicity* **XII**, 253–263 (1971).
2. A. M. Cohen and R. W. Ruddon, *Mol. Pharmacol.* **6**, 540–547 (1970).
3. J. Seifert and H. Remmer, *Biochem. Pharmacol.* **20**, 553–560 (1971).
4. R. T. Louis Ferdinand and G. C. Fuller, *Biochem. Biophys. Res. Commun.* **38**, 811–816 (1970).
5. M. J. Mycek, *Biochem. Pharmacol.* **20**, 325–337 (1971).
6. M. C. Lechner and C. R. Pousada, *Biochem. Pharmacol.* **20**, 3021–3028 (1971).
7. J. Seifert and J. Vácha, *Chem.-Biol. Interactions* **2**, 297–307 (1970).
8. J. Vácha and J. Seifert, *Biochem. Pharmacol.* **21**, 2156–2159 (1972).
9. H. Remmer, *Naturwissenschaften* **45**, 189–190 (1958).
10. A. H. Conney and J. J. Burns, *Nature* **184**, 363–364 (1959).
11. M. Ogur and G. Rosen, *Arch. Biochem. Biophys.* **25**, 262–276 (1950).
12. J. Řím an, J. Seifert, and J. Veselý, *Neoplasma* **5**, 379–395 (1958).
13. A. J. Munro, R. J. Jackson, and A. Korner, *Biochem. J.* **92**, 289–299 (1964).
14. K. S. Kirby, *Biochem. J.* **96**, 266–269 (1965).
15. J. D. Smith and R. Markham, *Biochem. J.* **46**, 509–513 (1950).
16. G. R. Wyatt, *Biochem. J.* **48**, 584–590 (1951).
17. H. Remmer and H. J. Merker, *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiedebergs)* **245**, 76–77 (1963).
18. S. W. Stäubli, R. Hess, and E. R. Weibel, *J. Cell Biol.* **42**, 92–112 (1969).
19. M. R. Gumbmann and S. N. Williams, *Biochem. Pharmacol.* **19**, 2861–2866 (1970).
20. C. A. Hirsch, *J. Biol. Chem.* **242**, 2822–2827 (1967).
21. W. Kunz, G. Schaude, H. Schimassek, W. Schmid, and M. Siess, *Proc. Eur. Soc. Study of Drug Toxicity* **VII**, 138–153 (1966).
22. T. S. Argyris, *J. Pharmacol. Exp. Ther.* **164**, 405–411 (1968).
23. T. S. Argyris and D. R. Magnus, *Develop. Biol.* **17**, 187–201 (1968).
24. S. Orrenius and J. L. E. Ericsson, *J. Cell Biol.* **28**, 181–198 (1966).