BBA 75766

STUDIES ON PLASMA MEMBRANES

XV. A SEX DIFFERENCE IN ALKALINE PHOSPHATASE ACTIVITIES OF PLASMA MEMBRANES ISOLATED FROM RAT LIVER

P. EMMELOT AND C. J. BOS

Department of Biochemistry, Antoni van Leeuwenhoekhuis, The Netherlands Cancer Institute, Amsterdam (The Netherlands)

(Received June 21st, 1971)

SUMMARY

1. The specific alkaline glycerolphosphatase activity (EC 3.1.3.1) of liver plasma membranes isolated from inbred male R-rats (young adults) was 4-fold higher than that of female liver membranes. Enzyme activity could be manipulated by castration and sex hormone administration, the non-hormone level being increased by testosterone and decreased by estrone treatment.

2. Various conditions which induce DNA synthesis and liver-cell proliferation increase plasma-membrane alkaline glycerolphosphatase, and it is suggested that, since the liver is an expanding tissue[1] and male rats grow faster than females, the sex difference in enzyme activity is a function of the latter process rather than the result of a direct regulatory effect of the sex hormones on the enzyme level *per se*.

3. The specific alkaline glycerolphosphatase activities of plasma membranes isolated from the transplanted rat hepatoma-484A, originally induced in a female R-rat, were similar for tumors grown in males and females. The liver-membrane enzyme is Zn^{2+} *plus* Mg^{2+} dependent with Ca^{2+} showing no activity, whereas Ca^{2+} may partially satisfy the ionic requirement of the hepatoma-membrane enzyme activity.

4. A similar sex (hormone) dependence, though quantitatively less pronounced than for the above enzyme, was observed for the alkaline nitrophenylphosphatase activity of the liver membranes. Some other plasma-membrane enzymes were apparently not sex dependent.

INTRODUCTION

As reported some years ago[2,3], plasma membranes isolated from rat liver in this laboratory exhibited no, or at the best only slight alkaline glycerolphosphatase activity (EC 3.1.3.1, substrate β-glycerol phosphate). Moderate to marked activities of the enzyme were, however, found in plasma membranes isolated from rat liver following prior bile-duct ligation[2], partial hepatectomy[3] or feeding of the hepatocarcinogen 4-dimethylaminoazobenzene[2]. Zero alkaline glycerolphosphatase activity has also been reported by COLEMAN AND FINEAN[4] for guinea-pig liver plasma mem-

branes. By contrast, mouse liver plasma membranes in our hands[5], demonstrated moderate alkaline glycerolphosphatase activity.

Our previous experiments with rats had mainly been carried out with females of the inbred strain R-Amsterdam, and hybrids between this strain and inbred strain U. In the present experiments it is shown that plasma-membrane alkaline phosphatases of R-rat liver are sex dependent, females showing low and males more pronounced activity.

MATERIALS AND METHODS

Throughout this investigation young adult male and female rats of the inbred strain R-Amsterdam were used. Starting 7 days following oophorectomy animals received subcutaneously hormone pellets, containing 2.5 mg testosterone propionate (daily except Sundays) or 0.2 mg estrone *plus* 1.8 mg cholesterol (once a week) for 3–4 weeks.

Plasma membranes were isolated from rat liver and rat hepatoma-484A as described previously[2,6]. For studying the metal dependence of the alkaline glycerolphosphatase, freshly isolated plasma membranes were suspended in 1 mM $NaHCO_3$ containing 1 mM EDTA, pH 7.4, for 10 min at 0°, reisolated by centrifugation for 10 min at 1500 × *g*, and then used for enzyme assay. In some experiments the liver membranes were pretreated with neuraminidase (EC 3.2.1.18); enzyme source (preparation b, of ref. 7) and procedure of treatment have been reported[7]. Enzyme assays were carried out according to methods previously used[2,8].

RESULTS

Alkaline glycerolphosphatase

Sex and sex hormone dependence. As shown in Table I, liver plasma membranes isolated from young adult male rats of inbred strain R-Amsterdam, about 3 months of age and weighing some 250 g, exhibited an approx. 4-fold higher specific enzyme activity (3.3–4.7 μmoles P_i released per mg membrane protein per h) than did the liver membranes obtained from females of corresponding age (0.8–1.3 μmoles P_i released). The latter animals weighed some 100 g less than did the males, but females of the corresponding weight of 250 g—being 1-year-old nonpregnant breeders—showed a similar low specific enzyme activity as did their younger virgin sisters.

Liver plasma membranes isolated from castrated males, which had been kept for 4–5 weeks following surgery, showed a 50 % drop in specific enzyme activity as compared with intact males. The enzyme activity of the membranes from castrated males was further depressed by estrone administration, *viz.* to one-half of the activity shown by the untreated castrates. This level of activity was exactly similar to that of the untreated females. Testosterone restored the reduced enzyme activity of the male castrates to near the level of intact males. Castration of females somewhat increased the membrane alkaline glycerolphosphatase activity. Testosterone administered to the castrated females caused a further small increase, whereas estrone resulted in a 3.4-fold decrease of the specific enzyme activity, the latter now being less than 50 % that of intact females.

The above results show that the alkaline glycerolphosphatase of rat-liver

plasma membranes is under sex hormone control, and suggest that the non-hormone enzyme level is enhanced by testosterone and reduced by estrone.

Possible relation to cell proliferation. We have previously found that bile-duct ligation, partial hepatectomy and feeding of the hepatocarcinogen 4-dimethylaminoazobenzene caused appreciable alkaline glycerolphosphatase activity to appear in the liver plasma membranes (for females see refs. 2 and 3; for males ref. 9, since, by that time we were unaware of the sex difference in enzyme activity, the enzyme of untreated male liver was not studied). All three procedures lead to DNA synthesis and cell proliferation in the liver; for bile-duct ligation see ref. 10. Hepatocarcinogens cause liver-cell regeneration (increased cell turnover) by being hepatotoxic. As shown in Table II, 2–4 weeks feeding of 4-dimethylaminoazobenzene to female rats more than doubled the specific activity of their liver-membrane alkaline glycerolphosphatase

TABLE I

ALKALINE GLYCEROLPHOSPHATASE OF LIVER PLASMA MEMBRANES ISOLATED FROM INTACT AND CASTRATED MALE AND FEMALE RATS AND CASTRATES TREATED WITH TESTOSTERONE OR ESTRONE

Rats were 3–4 months of age except in the experiments marked* in which 1-year-old females were used. Average specific enzyme activities and number of experiments are listed.

Sex	*Hormone treatment*	*μmoles P_i per mg protein per h*
♂, intact	None	3.8 ± 0.4 (7)
♀, intact	None	1.0 ± 0.2 (5)
♀, intact*	None	0.85 ± 0.05 (2)
♂, castrated	None	1.9 ± 0.2 (4)
♂, castrated	Testosterone	3.4 ± 0.1 (3)
♂, castrated	Estrone	0.9 ± 0.1 (3)
♀, castrated	None	1.35 ± 0.05 (3)
♀, castrated	Testosterone	1.85 ± 0.05 (3)
♀, castrated	Estrone	0.40 ± 0.01 (3)

TABLE II

EFFECT OF ADMINISTRATION OF HEPATOCARCINOGENS AND PHENOBARBITAL ON THE ALKALINE GLYCEROLPHOSPHATASE ACTIVITY OF ISOLATED PLASMA MEMBRANES FROM MALE AND FEMALE RAT LIVER

Standard diet throughout, unless indicated otherwise. 4-Dimethylaminoazobenzene was dissolved in rape oil and administered by stomachtube for 5 days per week. Dimethylnitrosamine and thioacetamide were dissolved in saline and injected in the tail vein and intraperitoneally, respectively.

Animal treatment	*μmoles P_i per mg protein per h*	
	Males	*Females*
Control, standard diet (range)	3.3–4.7	0.8–1.3
Polished rice diet (2–4 weeks)	—	0.75 ± 0.05 (3)
Polished rice diet and 4-dimethylaminoazobenzene (q.d. 10 mg for 2–4 weeks)	—	1.95 ± 0.15 (3)
Dimethylnitrosamine (once 50 mg/kg, 14–18 h)	4.4 ± 0.1 (3)	1.45 ± 0.05 (2)
Thioacetamide (once 30 mg/kg, 14 and 18 h)	3.6 ± 0.0 (2)	1.15 ± 0.05 (2)
Thioacetamide (once 30 mg/kg, 48 h)	6.4 ± 0.1 (2)	1.95 ± 0.05 (2)
Phenobarbital (1 g/l drinking water for 14 days)	2.2 ± 0.2 (2)	1.05 ± 0.15 (2)

in the present experiments. One dose of thioacetamide, which has been reported to increase DNA synthesis and mitosis in hepatocytes of Wistar rats at 36 h and later, also increased the alkaline glycerolphosphatase of liver plasma membranes obtained from male and female R-rats. By contrast, thioacetamide or dimethylnitrosamine for 14–18 h had no effect, as expected from the short time period involved; in fact these experiments were devised as control. Finally phenobarbital (in drinking water for 2 weeks), which may cause some DNA synthesis but mainly liver-cell hypertrophy[12,13], inhibited the male liver enzyme but had no effect in the case of the female; phenobarbital has been reported to inhibit also the 5'-nucleotidase of male rat liver and is further known to affect many other biochemical parameters of the liver cell[14].

Rat hepatoma plasma membranes. Hepatoma-484A originally induced in a female R-rat by 4-dimethylaminoazobenzene, was transplanted into male and female rats, and after 2 weeks the tumors were harvested and the plasma membranes isolated using 1 mM $NaHCO_3$ fortified with 2 mM $CaCl_2$ as homogenization medium[6]. The specific activities of the alkaline glycerolphosphatase exhibited by the hepatoma membranes from males and females were similar, *e.g.* 11.2 and 12.3 μmoles Pi released per mg protein per h, or slightly higher for females (Table III) in paired experiments. No difference in the growth rates of the tumors in males and females was noted.

Enzyme properties. Some 25 % of the protein of liver plasma membrane isolated in hypotonic bicarbonate, is derived from the cell sap (and intercellular fluid) and can be removed by suspending the membranes in 0.9 % NaCl (refs. 2,15). The alkaline glycerolphosphatase activity of the liver membranes of both males and females, either untreated or following administration of 4-dimethylaminoazobenzene or di-

TABLE III

EFFECT OF Mg^{2+}, Ca^{2+} AND Zn^{2+} ON ALKALINE GLYCEROLPHOSPHATASE ACTIVITY OF FRESH AND EDTA PRETREATED PLASMA MEMBRANES ISOLATED FROM RAT LIVER AND HEPATOMA

In the first experiment 0.1 mM Ca^{2+} and in Expts. 2–4 1 mM Ca^{2+} was used. The Zn^{2+} concentration in Expt. 5 was 10 μM, and 1 mM for values in parentheses. Some typical experiments are illustrated.

Membrane source	Mg^{2+}	*μmoles P_i per mg protein per h*			
		Fresh		*EDTA pretreated*	
		Minus Ca^{2+}	*Plus Ca^{2+}*	*Minus Ca^{2+}*	*Plus Ca^{2+}*
Liver ♂	+	3.9	4.1	0.9	0.8
	−	2.7	2.9	0.8	0.9
Liver ♂	+	4.5	4.5	1.3	1.5
Liver ♂	+	3.4	3.4	0.9	1.0
Hepatoma-484A ♂	+	12.4	12.1	3.9	7.2
♀	+	14.8	14.2	5.0	9.1
		Minus Zn^{2+}	*Plus Zn^{2+}*	*Minus Zn^{2+}*	*Plus Zn^{2+}*
Liver ♂	−	2.7	3.0 (1.4)	1.1	2.8 (1.2)
	+	4.1	4.2 (1.5)	1.4	4.0 (1.5)

methylnitrosamine, was for 95–100 % recovered in the saline-insoluble membrane portion, *i.e* the clean plasma membranes, indicating the membrane-bound nature of the enzyme.

The alkaline glycerolphosphatase is located in the liver plasma membranes lining the bile spaces. These membrane areas are coated by a globular repeat unit containing Co^{2+}-activated aminopeptidase activities, which is removable by mild papain digestion[9,16]. Liver plasma membranes from male and female rats were thus treated and the residual membranes separated from the globular knobs by high speed centrifugation[9]. All the alkaline glycerolphosphatase activity was recovered in the membrane fraction, while the globular units were essentially devoid of activity.

MIEDEMA[17] has reported that treatment of the HeLa cells with the enzyme neuraminidase (EC 3.2.1.18) which releases O-glycosidically linked sialic acid, increased the alkaline glycerolphosphatase activity on the surface of the cells. In 3 experiments with liver plasma membranes from male rats, the alkaline glycerolphosphatase was increased for 15, 20 and 23 % by the prior treatment of the membranes with neuraminidase (which released 70 % of the membrane sialic acid[2]) as compared with controls treated under similar conditions in the absence of the latter enzyme. In view of the possible presence of enzyme impurities in the neuraminidase preparation, not too much significance should be attributed to this result. However, on a comparative basis the similarity in reaction of the enzymes is interesting. Although isolated alkaline phosphatases from various sources[18,19] contain sialic acid, neuraminidase does not appear to affect the activity of the isolated enzyme[18]. The effect of neuraminidase on the membrane-bound enzyme may therefore be indirect, and, if involving membrane sialic acid, might be due to a better excess of substrate to enzyme, analogous to changes in other membrane properties following removal of sialic acid by neuraminidase (antigenicity[20], agglutinin receptors[21]).

The metal dependence of the alkaline glycerolphosphatase in liver plasma membranes has, to our knowledge, not been studied. The enzyme is commonly assayed – as in the present experiments unless indicated otherwise – in the presence of (8.4 mM) Mg^{2+}. In the absence of Mg^{2+} some 30 % inhibition of enzyme activity was noted (Table III). Pretreatment of the liver membranes with EDTA caused 70–75 % inhibition of the enzyme activity of the reisolated and washed membranes, whether or not Mg^{2+} was present during the assay. 1 mM Ca^{2+} had no effect on the alkaline glycerolphosphatase of the liver membranes under any of these conditions. By contrast, with plasma membranes isolated from rat hepatoma-484A, 1 mM Ca^{2+} (with Mg^{2+} present) partly restored the inhibition produced by EDTA. 10 μM Zn^{2+} completely restored the enzyme activity lost by EDTA pretreatment of the liver membranes as compared with the appropriate controls in the absence and presence of Mg^{2+}. Thus, the following sequence of ion capacity to restore alkaline glycerolphosphatase activity lost by EDTA pretreatment was observed (as compared with fresh membranes assayed in the presence of Mg^{2+}): 10 μM Zn^{2+} *plus* 8.4 mM Mg^{2+} (to 100 %) $>$ Zn^{2+} (70 %) $\gg$ Mg^{2+} (without virtual effect). This situation, including the inhibitory effect of higher Zn^{2+} concentration (1 mM, Table III), closely resembles that reported for isolated kidney alkaline glycerolphosphatase[22,23], and is compatible with the view that either two enzymes are present in both preparations (one dependent on Mg^{2+} and the other not, while both are Zn^{2+} dependent) or that one Zn^{2+}-dependent enzyme preferably hydrolyzes the magnesium salt of β-glycerol phosphate[23].

TABLE IV

ALKALINE NITROPHENYLPHOSPHATASE, K^+-ACTIVATED NITROPHENYLPHOSPHATASE, AND ACID NITROPHENYLPHOSPHATASE OF LIVER PLASMA MEMBRANES ISOLATED FROM INTACT AND CASTRATED MALE AND FEMALE RATS, AND CASTRATES TREATED WITH TESTOSTERONE OR ESTRONE

Figures in parentheses illustrate range.

Sex (number of experiments)	*Hormone treatment*	*μmoles P_i per mg protein per h*		
		Alkaline nitrophenyl-phosphatase	*Alkaline K^+-nitrophenyl-phosphatase*	*Acid nitrophenyl-phosphatase*
♂, intact (4)	None	3.31 ± 0.2 (2.9–3.7)	1.6 ± 0.3 (1.1–1.9)	9.1 ± 1.3 (7.5–10.3)
♀, intact (3)	None	1.97 ± 0.04 (1.9–2.0)	1.6 ± 0.5 (1.0–2.3)	7.3 ± 0.33 (6.8–7.7)
♂, castrated (5)	None	2.30 ± 0.21 (2.0–2.7)	1.7 ± 0.4 (1.1–2.3)	8.6 ± 1.3 (7.3–10.2)
♂, castrated (3)	Testosterone	2.63 ± 0.04 (2.6–2.7)	2.2 ± 0.3 (1.8–2.6)	9.9 ± 0.6 (9.0–10.6)
♂, castrated (3)	Estrone	1.75 ± 0.03 (1.7–1.8)	1.6 ± 0.4 (1.0–2.0)	7.7 ± 0.2 (7.5–8.0)
♀, castrated (2)	None	2.2 ± 0.0	1.9 ± 0.5	8.1 ± 0.7
♀, castrated (2)	Testosterone	2.85 ± 0.05	2.1 ± 0.1	8.5 ± 0.0
♀, castrated (2)	Estrone	1.55 ± 0.05	2.05 ± 0.05	8.7 ± 0.3

Alkaline nitrophenylphosphatase

Isolated rat-liver plasma membranes also hydrolyze *p*-nitrophenyl phosphate, the enzyme involved being probably not the same as that hydrolyzing β-glycerol phosphate[2]. The specific alkaline nitrophenyl phosphate of male liver plasma membranes was some 70 % higher than that of the female ones (Table IV). Castration of males caused a small but significant drop in enzyme activity[2], the latter activity being now similar to that of female castrates. Castrated males and females treated with testosterone exhibited higher membrane enzyme activity (50 and 80 %, respectively) than did the corresponding castrates treated with estrone. Thus, the plasma membrane alkaline nitrophenyl phosphatase behaved similar to the glycerolphosphatase, though quantitatively less outspoken, in respect of sex and reaction to sex-hormone administration. No such dependence was (readily or generally) apparent from the data obtained on the K^+-activated alkaline nitrophenylphosphatase and the acid nitrophenylphosphatase (Table IV), or the 5'-mononucleotidase (substrate: AMP; not illustrated).

DISCUSSION

The alkaline glycerolphosphatase is located in the plasma membranes lining the bile spaces, and isolated rat-liver plasma membranes are rich in bile canalicular structures[2,15]. If these membranes were more fragile in the case of the female than of the male liver, leading to fragmentation and fractional loss during preparation, the present results of a sex difference in membrane enzyme activity could be interpreted accordingly. This is, however, a very faint possibility, since no support for this view has been obtained electron microscopically, and another enzyme, *i.e.* 5'-mono-

nucleotidase, which is also concentrated in the membranes lining the bile canaliculi (*cf.* ref. 2), was not sex dependent. The latter finding also discourages the view that sex hormones in some way affect the size of the bile-space surface area relative to that of the total plasma-membrane skeleton. Thus a more direct control of sex hormones over the plasma-membrane alkaline glycerolphosphatase activity or level is indicated. Other instances of such control of membrane-bound enzymes in the liver are known, *e.g.* the drug-metabolizing enzymes of the microsomal membranes[24]. Although BITENSKY *et al.*[25] have found that the adenyl cyclase activity displayed by liver homogenate and crude fractions is sex (hormone) dependent, whereas others have shown this enzyme to be present in plasma membranes[26,27], the present findings on the alkaline phosphatases furnish the first direct evidence of such a relationship for a plasma-membrane enzyme. Our data allow the conclusion that the nonhormone (castrate) level of enzyme activity is increased by testosterone and decreased by oestrone. As regards the mechanism by which these effects are obtained, it is of interest that various conditions which induce DNA synthesis and cell proliferation (turnover) in liver, increased also the alkaline glycerolphosphatase activity of the liver-plasma membranes of sexually intact rats. This is in line with an old observation that growing tissues (especially tumors) may contain elevated levels of alkaline phosphatase[23,28]. Recently, the temporal relationship of the alkaline phosphatase activity to the DNA synthetic phase has been established for HeLa cells[29]. These cells contain the enzyme in their surface, and this enzyme[17] reacted to neuraminidase in the same way as the liver-plasma alkaline glycerolphosphatase did. The possibility that sex hormones do not act directly on the processes of synthesis or breakdown of the liver-membrane alkaline phosphatase *per se*, but may act indirectly by affecting liver growth (liver-cell proliferation) with testosterone being stimulatory and estrone inhibitory, should, therefore, not be excluded. Liver is a socalled expanding tissue[1,30], increasing in mass until rats are 1 year of age, and growth proceeds faster in males than in females.

Finally, alkaline glycerolphosphatase appears to be concentrated in plasma-membrane areas specialized for transport[16], *e.g.* those lining the bile canaliculi in liver and the brush borders of the intestine and kidney proximal tubuli. The liver plasma membrane enzyme is $Zn^{2+} + Mg^{2+}$ dependent, as is the isolated kidney enzyme and possibly also the intestinal enzyme[23]. It would be of interest to investigate whether the latter enzyme activities are also sex dependent. The relative similarity in differentiation of these membrane transport areas has previously been stressed[16]: the alkaline glycerolphosphatase appears to be a component of the membrane element *per se*, whereas the 50–60-Å globular knobs coating these membranes contain Co^{2+}-activated aminopeptidase activities.

REFERENCES

1 R. J. GOSS, in H. TEIR AND T. RYTÖMAA, *Control of Cellular Growth in Adult Organisms*, Academic Press, New York, 1967, p. 3.
2 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND P. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.
3 P. EMMELOT AND E. L. BENEDETTI, in *Carcinogenesis: A Broad Critique*, M.D. Anderson Hosp. Tumor Inst. Symp. Univ. of Texas, Williams and Wilkins, Baltimore, Md., 1967, p. 471.
4 R. COLEMAN AND J. B. FINEAN, *Biochim. Biophys. Acta*, 125 (1966) 197.
5 P. EMMELOT AND C. J. BOS, *Int. J. Cancer*, 4 (1969) 723.
6 P. EMMELOT AND C. J. BOS, *Int. J. Cancer*, 4 (1969) 705.
7 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 150 (1968) 341.

8 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 121 (1966) 375.
9 P. EMMELOT, A. VISSER AND E. L. BENEDETTI, *Biochim. Biophys. Acta*, 150 (1968) 364.
10 T. A. LAWSON AND F. K. DZHIOEV, *Chem.-Biol. Interactions*, 2 (1970) 165.
11 J. REDDY, M. CHIGA AND D. SVOBODA, *Lab. Invest.*, 20 (1969) 405.
12 T. ARGYRIS, *J. Pharmacol. Exp. Therap.*, 164 (1968) 405.
13 W. STÄUBLI, R. HESS AND E. R. WEIBEL, *J. Cell Biol.*, 42 (1969) 92.
14 J. SEIFERT AND J. VÁCHA, *Chem.-Biol. Interactions*, 2 (1970) 297.
15 E. L. BENEDETTI AND P. EMMELOT, in A. J. DALTON AND F. HAGUENAU, *The Membranes*, Academic Press, New York, 1968, p. 33.
16 P. EMMELOT AND A. VISSER, *Biochim. Biophys. Acta*, 241 (1971) 273.
17 E. MIEDEMA, *Exp. Cell Res.*, 53 (1968) 488.
18 S. SARASWATHI AND B. K. BACHHAWAT, *Biochim. Biophys. Acta*, 212 (1970) 170.
19 J. C. ROBINSON AND J. E. PIERCE, *Nature*, 204 (1964) 472.
20 G. A. CURRIE AND K. D. BAGSHAWE, *Br. J. Cancer*, 22 (1968) 843.
21 M. M. BURGER, in L. A. MANSON, *Biological Properties of Mammalian Surface Membrane*, The Wistar Inst. Press, 1968, p. 78.
22 J. C. MATHIES, *J. Biol. Chem.*, 233 (1958) 1121.
23 L. A. HEPPEL, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 5, Academic Press, New York, 1961, p. 49.
24 G. P. QUINN, J. AXELROD AND B. B. BRODIE, *Biochem. Pharmacol.*, 1 (1958) 1952.
25 M. W. BITENSKY, V. RUSSELL AND M. BLANCO, *Endocrinology*, 86 (1970) 154.
26 S. L. POHL, L. BIRNBAUMER AND M. RODBELL, *J. Biol. Chem.*, 246 (1971) 1849.
27 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 249 (1971) 285.
28 S. EDLBACHER AND W. KUTSCHER, *Z. Physiol. Chem.*, 199 (1931) 200.
29 J. R. CHURCHILL AND G. P. STUDZINSKI, *J. Cell Physiol.*, 75 (1970) 297.
30 C. P. LEBLOND, in *Control of Cell Division and the Induction of Cancer, Natl. Cancer Inst. Monograph*, 14 (1964) 119.