Biochemical Pharmacology, Vol. 22, pp. 1557–1564. Pergamon Press, 1973. Printed in Great Britain.

INFLUENCE OF FASTING AND HEMIN ON MICROSOMAL CYTOCHROMES AND ENZYMES*

KARL W. BOCK, WOLFGANG FRÖHLING and HERBERT REMMER
Institut für Toxikologie der Universität Tübingen, West Germany

(*Received* 31 *October* 1972; *accepted* 2 *January* 1973)

Abstract—During fasting for 24 hr the amount of microsomal cytochrome P-450 and cytochrome b_5 are reduced by 30 and 50 per cent, respectively, whereas microsomal glucose-6-phosphatase and NAD glycohydrolase remain unchanged when compared on a whole liver basis. Intraperitoneally injected hemin reduces microsomal cytochromes in fed and fasted rats. It suppresses the induction of cytochrome P-450, NADPH-cytochrome *c* reductase and UDP glucuronyltransferase by phenobarbital. In contrast, microsomal glucose-6-phosphatase and NAD glycohydrolase are not affected by phenobarbital or hemin. The effects of hemin are only transient, probably due to the induction of liver microsomal heme oxygenase (Tenhunen *et al.*[24]). Judged from the rate of loss of radioactivity in purified cytochrome b_5 prelabeled in the heme moiety with ^{3}H-δ-amino-levulinic acid, the decrease of cytochromes after hemin treatment is due to a decreased synthesis as well as increased breakdown.

INTRAPERITONEALLY injected hemin reduces the level of mitochondrial δ-aminolevulinic acid synthetase and suppresses the induction† of cytochrome P-450 by phenobarbital.[1,2] Heme synthesis seems to be regulated primarily at the level of δ-aminolevulinic acid synthetase.[3] This enzyme is induced during the induction of cytochrome P-450 by phenobarbital in fasting rats[2] but not in fed animals.[4] In addition, during the induction process cytochrome P-450 is increased to a higher level in fasting than in fed rats.[4,5] Based on these findings the influence of hemin on microsomal cytochromes was followed separately in fed and fasting animals. The aim of our studies was to clarify the influence of hemin on the regulation of microsomal cytochromes and to gain some insight into a possible correlation between the induction of cytochrome P-450, NADPH-cytochrome *c* reductase (EC 1.6.2.3) and UDP glucuronlytransferase (EC 2.4.1.17). These proteins are firmly bound to liver endoplasmic reticulum membranes. Glucose-6-phosphatase (EC 3.1.3.9) and NAD glycohydrolase (EC 3.2.2.6) were followed as enzymes not functionally related to mixed function oxidase but bound to the same membranes.

METHODS AND MATERIALS

Treatment of animals. Male Sprague–Dawley rats (150–200 g) were fed *ad lib.* on a standard diet containing 20% protein (Altromin-R, Lage-Lippe, Germany). During

* Presented in parts at the Fifth International Congress on Pharmacology, San Francisco, 1972 and at the Eighth FEBS Meeting, Amsterdam, 1972.

† Induction is used in the operational definition discussed by Greengard[6] as any increase in the amount of a given enzyme regardless of the mechanism causing the increase.

starvation the animals were kept in wire cages. Phenobarbital was given by a single intraperitoneal injection of 100 mg/kg. Hemin (16 mg/kg) was injected as a 2·5 mM methemealbumin solution 2 hr before, together with and every 12 hr after the injection of phenobarbital. The methemealbumin solution was prepared as described by Tenhunen *et al.*[7] Identical results were obtained when hemin was injected without albumin.

Preparation of microsomal fractions and purification of microsomal cytochrome b_5. Livers were perfused through the portal vein with 0·9% NaCl (w/v) and excised under ether anesthesia, minced with scissors and homogenized in 4 vol. of 0·25 M sucrose with a motor driven homogenizer with Teflon pestle. The homogenate was centrifuged at 10,000 *g* for 15 min to sediment the nuclei and mitochondria, the ensuing supernatant was spun at 100,000 *g* for 45 min and the sediment washed with 0·15 M KCl + 25 mM Tris–HCl, pH 7·4 by resuspension and resedimentation. The final pellet was suspended in 0·25 M sucrose. Cytochrome b_5 was purified from rat liver microsomes as described by Omura *et al.*[8]

Enzyme assays and chemical determinations. Spectra were recorded with an Aminco-Chance duel wavelength/split beam or with the Leitz-Unicam SP 800 recording spectrophotometer. Cytochrome P-450 and cytochrome b_5 in liver microsomes were determined with the method of Omura and Sato.[9] In the presence of contaminating hemoglobin, e.g. in liver homogenates, cytochrome P-450 was assayed as described by Greim[5] and Schoene *et al.*[10] Identical results were obtained with both methods in liver microsomes. Purified cytochrome b_5 was determined by its absolute spectrum.[11] Total heme was extracted from microsomes with 2-butanone–HCl as described by Bock and Siekevitz[12] and determined by a pyridine hemochromogen method.[9]

NAD glycohydrolase was assayed as described previously.[13] Glucose-6-phosphatase was determined according to Swanson[14] while NADPH-cytochrome *c* reductase was measured as described by Dallner *et al.*[15] at 22°. UDP glucuronyltransferase was tested in Triton X-100 activated microsomes as discussed by Mulder[16] by a modification of the procedure of Hollmann and Touster.[17] The incubation mixture consisted of 1mM *p*-nitrophenol, 2 mM UDP glucuronic acid, 50 mM Tris–HCl, pH 7·4, 0·2% (w/v) Triton X-100, 5 mM $MgCl_2$ and 1 mg microsomal protein in a total volume of 1 ml. The reaction was linear up to 6 min. Protein was determined with the method of Lowry *et al.*[18] using bovine serum albumin as a standard. Phospholipid was extracted from microsomes by the procedure of Folch *et al.*[19] using 0·73% (w/v) NaCl as the aqueous phase. Inorganic phosphate was assayed by the method of Ames and Dubin,[20] and micromoles of phosphorus were multiplied by 775 to obtain micrograms of phospholipid. Radioactivity was determined by liquid scintillation counting in Bray's solution[21] and quenching was monitored by the addition of an internal standard.

Materials. δ-Aminolevulinic acid-3,5-^{3}H was obtained from New England Nuclear Corp., Boston, Mass., and crystalline bovine hemin (Type 1) from Sigma Chemical Corp., St. Louis, Missouri.

RESULTS

Influence of fasting on the amount of liver microsomal cytochromes. Liver wet weight, total protein as well as the amount of endoplasmic reticulum membranes varied considerably during fasting or treatment with drugs. Therefore changes in microsomal proteins should be compared on a whole liver basis as well as on the basis of specific

activity. From the content of cytochrome P-450 per gram of liver wet weight and its amount per milligram of microsomal protein the amount of microsomal protein per gram of liver wet weight was calculated. With this operational value the amounts of microsomal cytochromes and enzymes were calculated on a whole liver basis. A slightly higher value of microsomal protein was obtained with glucose-6-phosphatase which is also found solely in endoplasmic reticulum membranes.[22,23]

TABLE 1. INFLUENCE OF FASTING ON CYTOCHROME P-450, GLUCOSE-6-PHOSPHATASE AND NAD GLYCOHYDROLASE COMPARED ON A WHOLE LIVER BASIS

	Period of fasting (days)			
	0	1	2	3
Body weight (g)	170 ± 12	155	140	129
Liver wet weight (g)	8·1 ± 1	5·5	4·6	4·7
Cytochrome P-450				
(nmoles/g liver)	29 ± 4	29	38	39
(nmoles/mg microsomal protein)	0·93 ± 0·05	0·96	1·10	1·26
Glucose-6-phosphatase				
(μmoles/min/g/ liver)	11·5 ± 2	20	21	
(μmoles/min/mg microsomal protein)	0·28 ± 0·03	0·46	0·49	0·45
NAD glycohydrolase				
(μmoles/min/mg microsomal protein)	0·026 ± 0·003	0·040	0·040	0·035
Microsomal protein (mg/g liver)				
calculated from cytochrome P-450*	31 ± 4	30	35	31
calculated from glucose-6-phosphatase*	41 ± 6	43	43	
Cytochrome P-450/whole liver†	235 ± 39	159	177	184
Glucose-6-phosphatase/whole liver†	70 ± 11	76	79	66
NAD glycohydrolase/whole liver†	6·5 ± 0·5	6·6	6·4	5·1

* $\frac{\text{Content or activity/gram of liver}}{\text{Content or activity/milligram of microsomal protein}}$.

† Content or activity/milligram microsomal protein × microsomal protein (calculated from cytochrome P-450)/gram of liver × liver wet weight.

Treatment of rats, preparation of microsomes and assays were performed as described in Methods and Materials. The values are averages of four experiments. Two rats were used for each time point. Control values ± S.D. were taken from four to ten determinations.

As shown in Table 1 the content of cytochrome P-450 per gram of liver or per milligram of microsomal protein, seems to be unchanged or slightly elevated during fasting. However, on a whole liver basis the cytochrome is reduced. On the other hand, glucose-6-phosphatase and NAD glycohydrolase per milligram of microsomal protein seem to be elevated during fasting, whereas the levels are virtually unchanged on a whole liver basis.

Influence of hemin and phenobarbital on the amount of liver microsomal cytochromes, NADPH-cytochrome c reductase and UDP glucuronyltransferase. The influence of hemin on the synthesis of microsomal cytochromes was studied on a whole liver basis, separately in fed (Fig. 1a) and in fasting (Fig. 1b) rats. The induction of cytochrome P-450 by phenobarbital is different under these nutritional states. The levels of cytochrome P-450 and cytochrome b_5 as well as the induction of cytochrome P-450 by phenobarbital are reduced by hemin treatment. Note that the inhibition of cytochrome P-450 induction is reduced with time despite continuous treatment with hemin.

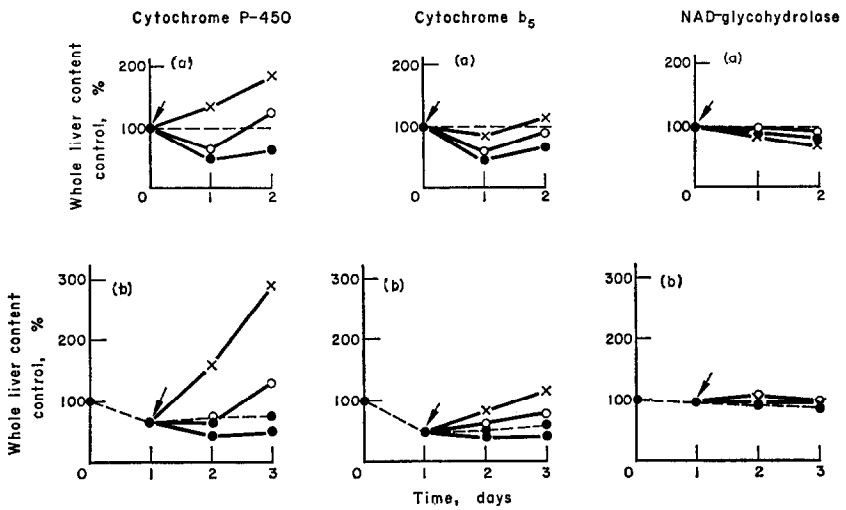

FIG. 1. Influence of hemin and phenobarbital on microsomal cytochromes in fed (a) and fasting (b) rats. ●- - -● Controls; ×——× treatment with 100 mg/kg phenobarbital at the time indicated by the arrow; ○——○ hemin treatment (16 mg/kg) 2 hr before, together with and every 12 hr after phenobarbital; ●——● hemin treatment without phenobarbital. 100 per cent values for cytochrome P-450 and NAD glycohydrolase are given in Table 1. For microsomal cytochrome b_5 141 ± 24 nmoles per whole liver (calculated as described in Table 1) were taken as 100 per cent. The averages of three experiments are listed.

NAD glycohydrolase which is also bound to endoplasmic reticulum membranes is not affected by treatment of rats with hemin, phenobarbital or fasting of the animals. Only during phenobarbital treatment of fed rats was a slight decrease of the enzyme seen. The stability of NAD glycohydrolase during fasting correlates with its slow apparent turnover under steady state conditions.[13] Likewise glucose-6-phosphatase was not affected by hemin (Table 2). Furthermore, the ratio of phospholipids to proteins in microsomal membranes (0·4–0·5) is constant during the experiments given in Fig. 1. Only 24 hr after phenobarbital treatment this ratio was increased to 0·6 indicating that the amount of phospholipids exceeds the synthesis of proteins initially during the proliferation of endoplasmic reticulum membranes by phenobarbital.

TABLE 2. INFLUENCE OF HEMIN ON LIVER MICROSOMAL CYTOCHROME P-450 AND GLUCOSE-6-PHOSPHATASE

	Control	Hemin treatment
Liver wet weight (g)	7·8	6·0
Cytochrome P-450		
(nmoles/mg microsomal protein)	0·96	0·57
(nmoles/whole liver)*	240	120
Glucose-6-phosphatase		
(μmoles/min/mg microsomal protein	0·28	0·32
(μmoles/min/whole liver)*	70	67

The data are taken from experiments described in Fig. 1. Hemin (16 mg/kg) was injected intraperitoneally into rats 26, 24 and 12 hr before sacrifice. Averages of three experiments are listed.
* Calculated as described in Table 1.

Microsomal cytochrome *c* reductase and UDP glucuronyltransferase which are functionally linked to cytochrome P-450 respond to treatment with phenobarbital and hemin in a way similar to cytochrome P-450 although to a lesser extent (Fig. 2).

Influence of hemin on synthesis and breakdown of microsomal cytochrome b_5. In order to elucidate whether the decrease of cytochrome P-450 and cytochrome b_5 during hemin treatment is caused by a diminished synthesis rate or an accelerated breakdown the heme moiety was prelabeled with ^{3}H-δ-aminolevulinic acid. The decrease of the label was followed in purified cytochrome b_5 and in total microsomal hemes.

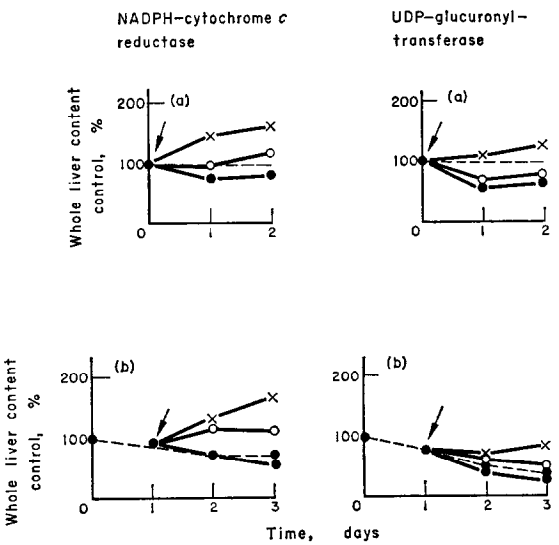

FIG. 2. Influence of hemin and phenobarbital on microsomal NADPH-cytochrome *c* reductase and UDP glucuronyltransferase. The enzymes were determined in microsomes from experiments given in Fig. 1. For NADPH-cytochrome *c* reductase 8·2 ± 1·6 μmoles/min/whole liver and for UDP glucuronyltransferase 8·9 ± 1·7 μmoles/min/whole liver were taken as 100 per cent. The averages of three experiments are listed.

After injection of ^{3}H-δ-aminolevulinic acid into rats the specific radioactivity of heme in cytochrome b_5 rose up to 24 hr. It then decreased exponentially with a half-life time of approx. 2 days.[5,12] Therefore, hemin treatment started 24 hr after the injection of ^{3}H-δ-aminolevulinic acid. If hemin treatment stopped completely the intracellular heme synthesis (and concomitantly the synthesis of cytochrome b_5), no difference in the total amount of prelabeled cytochrome b_5 molecules in the liver of controls and hemin treated rats could be observed, because the incorporation of radioactive precursor ended when hemin began to act suppressively. However, the specific radioactivity of microsomal cytochrome b_5 in the liver of rats receiving hemin would be higher if hemin inhibited heme synthesis, since less unlabeled cytochrome molecules are incorporated into microsomes. If, on the other hand, only the degradation of cytochrome b_5 increased during administration of hemin, the amount as well as the specific radioactivity of labeled cytochrome b_5 would be lower than in controls.

The experiments listed in Table 3 indicate that an increased breakdown as well as an inhibited synthesis of cytochrome b_5 occurred. The lower amount of labeled

cytochrome b_5 molecules in the whole liver signified an enhanced degradation. However, the decrease was not accompanied by a comparable decline in the specific radioactivity, indicating that hemin also inhibits heme synthesis.

TABLE 3. INFLUENCE OF HEMIN ON THE LOSS OF RADIOACTIVITY IN MICROSOMAL CYTOCHROME b_5 PRELABELED IN ITS HEME MOIETY

	Control	Hemin treatment
Cytochrome b_5		
(nmoles/mg microsomal protein)	0·52 ± 0·04	0·29 ± 0·03
(nmoles/whole liver)*	141 ± 24	65 ± 6
dis/min/nmole	404 ± 36	451 ± 70
dis/min/whole liver*	57,000 ± 6000	29,300 ± 8700
Microsomal heme		
dis/min/nmole	323 ± 37	261 ± 37

Rat liver microsomal cytochrome b_5 was prelabeled in its heme moiety by injecting 10 μCi δ-aminolevulinic acid-3,5-^{3}H (1000 μCi/mmole) intraperitoneally. 22, 24 and 36 hr later 16 mg/kg hemin was injected intraperitoneally and the animals were sacrificed 48 hr after labeling. Preparation of microsomes, purification of cytochrome b_5 and extraction of total microsomal hemes were performed as described in Methods and Materials. The mean ± S.D. of four experiments are listed. Determinations of cytochrome P-450 in homogenates and in microsomes were used for the calculation of cytochrome b_5 on a whole liver basis.
* Calculated as described in Table 1.

We therefore assume that the number of radioactive cytochrome b_5 molecules 22 hr after injection of δ-aminolevulinic acid before hemin treatment should be equal in controls and hemin treated rats, and amounts to about 80,000 dis/min in the whole liver (extrapolated graphically if the half-life of 2 days determined for the heme of cytochrome b_5 is taken into account). The amount of cytochrome b_5 falls to 57,000 dis/min in the liver of controls during the following 24 hr (Table 3). But only 29,000 dis/min of cytochrome b_5 was found in the liver of rats treated with hemin. We take this to mean that the breakdown of the prelabeled cytochrome b_5 is doubled during hemin administration.

Assuming an unaltered synthesis rate (together with an increased breakdown) the specific radioactivity of cytochrome b_5 must be lower in hemin treated rats. In this case less labeled cytochrome b_5 molecules are present, but dilution with newly synthesized unlabeled molecules would be the same in both groups. Our data does not allow this assumption because the specific radioactivity is nearly the same in controls and hemin treated rats, presenting clear cut evidence that decreased synthesis also contributes to the decline of cytochrome b_5.

It can be reasonably concluded that the decrease of cytochrome P-450 after hemin treatment is also caused by reduced synthesis as well as increased breakdown.

DISCUSSION

An attempt was made to follow liver microsomal proteins on a whole liver basis during fasting or treatment with drugs. The amount of total microsomal protein per liver was calculated from the content of cytochrome P-450 per liver and its specific content in microsomes. The value of microsomal protein changed with the method

used for preparing the microsomes. Nevertheless a value of microsomal protein was useful for comparative studies providing the microsomes are prepared in the same manner. Since liver wet weight and total protein change markedly during fasting or treatment with drugs a comparison of microsomal cytochromes or enzymes per gram of liver wet weight or per milligram of microsomal protein can be misleading (Table 1). Only when the cytochromes are compared on a whole liver basis does their marked decrease after 1 day of fasting become obvious. When the amount of microsomal protein was calculated from another exclusive microsomal constituent, glucose-6-phosphatase, its value was slightly higher. However, when the liver content of microsomal cytochromes was compared on the basis of glucose-6-phosphatase a similar decrease of microsomal cytochromes could be seen. The measurement of distinct spectrum of cytochrome P-450 may be more accurate than the determination of glucose-6-phosphatase, since glucose-6-phosphate is split to a minor degree by unspecific phosphatases. Furthermore the estimation of enzyme amounts from activity measurements are subject to some error. An activation during fasting has been described for glucose-6-phosphatase[26] and NAD glycohydrolase.[27] When enzyme activity is expressed as per milligram of liver protein or per gram of liver wet weight a spurious increase in activity can be expected since during fasting total liver proteins decrease and a "concentration" of liver tissue occurs due to the breakdown of large glycogen areas. On the other hand, activity and stability of glucose-6-phosphatase and UDP glucuronyltransferase are drastically changed by altering membrane structure.[28,29] Since in Table 1 the increase in activity of glucose-6-phosphatase fairly corresponds to the decrease in liver weight the contribution of an "activation" to the enzyme activity may be low.

The rapid decrease of microsomal cytochromes during fasting is not fully understood. It may be important to take it into account since in many studies on microsomal cytochromes rats are starved 1 day before preparing the microsomes. It correlates with reduced drug metabolism during fasting.[30] Phenobarbital induces the drug hydroxylating enzyme system (cytochrome P-450, NADPH-cytochrome *c* reductase) as well as UDP glucuronyltransferase. The question arises if the synthesis of these proteins is correlated or if they are induced independently. A specific inhibitor of heme and consequently cytochrome synthesis would help to clarify this problem.

Hemin decreases the level of cytochromes and suppresses the induction of cytochrome P-450 by phenobarbital by inhibiting heme synthesis at the level of δ-aminolevulinic acid synthetase.[1,2] The response of NADPH-cytochrome *c* reductase and UDP glucuronyltransferase to phenobarbital as well as phenobarbital plus hemin in fed and fasting rats indicates that the synthesis of these proteins is correlated with that of cytochrome P-450 (Figs. 1 and 2). However, our studies with prelabeled cytochrome b_5 also point to an increase of the breakdown of cytochromes by hemin. There are several possibilities for this increased breakdown. (a) Part of this breakdown may be due to rats being treated with hemin, as after this treatment they do not eat as much as untreated controls and lose liver weight in a way similar to fasted animals. (b) A direct interaction of hemin with microsomal membranes is indicated by the presence of a heme oxygenase system which is induced after hemin treatment.[24,25] The induction of this system may be responsible for the transient nature of the inhibitory effect of hemin on the induction of cytochrome P-450 by phenobarbital. (c) Lipid peroxidation which is stimulated by chelated iron compounds is well known in liver

microsomes.[31,32] However, the extent of lipid peroxidation in endoplasmic reticulum membranes in the intact cell is not known since factors present in the cytoplasma inhibit lipid peroxidation.[33] Gross lipid peroxidation damage after hemin treatment can be excluded since the phospholipid to protein ratio as well as glucose-6-phosphatase activity are not changed after hemin treatment (Table 2). It is known that glucose-6-phosphatase is particularly sensitive to lipid peroxidation.[34]

Hemin effectively inhibits the synthesis of heme and microsomal cytochromes. Since it also increases the breakdown of these cytochromes and since its inhibitory effect is only transient its value as an inhibitor of heme synthesis is limited.

Acknowledgements—The authors are indebted to Mrs. E. Wiegand for competent technical assistance and to the Deutsche Forschungsgemeinschaft for financial support.

REFERENCES

1. H. S. MARVER, R. SCHMID and H. SCHÜTZEL, *Biochem. biophys. Res. Commun.* **33,** 969 (1968).
2. H. S. MARVER, in *Microsomes and Drug Oxidations* (Eds. J. R. GILLETTE, A. H. CONNEY, C. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS and G. J. MANNERING), p. 495. Academic Press, New York (1969).
3. S. GRANICK, *J. biol. Chem.* **241,** 1359 (1966).
4. K. W. BOCK, E. KRAUSS and W. FRÖHLING, *Eur. J. Biochem.* **23,** 366 (1971).
5. H. GREIM, *Archs. Pharmak.* **266,** 261 (1970).
6. O. GREENGARD, *Adv. Enzyme Reg.* **5,** 397 (1967).
7. R. TENHUNEN, H. S. MARVER and R. SCHMID, *Proc. natn. Acad. Sci. U.S.A.* **61,** 748 (1968).
8. T. OMURA, P. SIEKEVITZ and G. E. PALADE, *J. biol. Chem.* **242,** 2389 (1967).
9. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370, 2379 (1964).
10. B. SCHOENE, R. A. FLEISCHMANN, H. REMMER and H. F. V. OLDERSHAUSEN, *Eur. J. clin. Pharmac.* **4,** 65 (1972).
11. T. OMURA and S. TAKESUE, *J. Biochem.* **67,** 249 (1970).
12. K. W. BOCK and P. SIEKEVITZ, *Biochem. biophys. Res. Commun.* **41,** 374 (1970).
13. K. W. BOCK, P. SIEKEVITZ and G. E. PALADE, *J. biol. Chem.* **246,** 188 (1971).
14. M. A. SWANSON, *J. biol. Chem.* **184,** 647 (1950).
15. G. DALLNER, P. SIEKEVITZ and G. E. PALADE, *J. Cell. Biol.* **30,** 97 (1966).
16. G. J. MULDER, *Biochem. J.* **117,** 319 (1970).
17. S. HOLLMANN and O. TOUSTER, *Biochim. biophys. Acta* **62,** 338 (1962).
18. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
19. J. FOLCH, M. LEES and G. H. S. STANLEY, *J. biol. Chem.* **226,** 497 (1957).
20. B. N. AMES and D. T. DUBIN, *J. biol. Chem.* **235,** 769 (1960).
21. G. A. BRAY, *Analyt. Biochem.* **1,** 279 (1960).
22. C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX and F. APPELMANS, *Biochem. J.* **60,** 604 (1955).
23. L. E. TICE and R. J. BARRNETT, *J. Histochem. Cytochem.* **10,** 754 (1962).
24. R. TENHUNEN, H. S. MARVER and R. SCHMID, *J. biol. Chem.* **244,** 6388 (1969).
25. R. TENHUNEN, H. S. MARVER and R. SCHMID, *J. Lab. Clin. Med.* **75,** 410 (1970).
26. R. C. NORDLIE, W. J. ARION, T. L. HANSON, J. R. GILSDORF and R. N. HORNE, *J. biol. Chem.* **243,** 1140 (1968).
27. R. L. BLAKE, S. L. BLAKE and E. KUN, *Biochim. biophys. Acta* **148,** 293 (1967).
28. D. ZAKIM, *J. biol. Chem.* **245,** 4953 (1970).
29. D. A. VESSEY and D. ZAKIM, *J. biol. Chem.* **246,** 4649 (1971).
30. R. KATO and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **150,** 279 (1965).
31. A. L. TAPPEL and H. ZALKIN, *Archs. Biochem. Biophys.* **80,** 333 (1959).
32. P. HOCHSTEIN, K. NORDENBRAND and L. ERNSTER, *Biochem. biophys. Res. Commun.* **14,** 323 (1964).
33. A. M. GRIMWADE, M. E. LAWSON and G. S. BOYD, *Biochem. J.* **126,** 14 P (1971).
34. E. D. WILLS, *Biochem. J.* **123,** 983 (1971).