*SEPARATE GENES DETERMINING THE STRUCTURE AND INTRACELLULAR LOCATION OF HEPATIC GLUCURONIDASE**

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A major factor influencing the contribution of an enzyme to the economy of the cell in which it resides is the site at which the enzyme is located within the cell. An important question then is whether the predilection of enzymes for certain intracellular sites derives only from the chemical structure of the enzyme or whether additional and specific enzyme localizing factors occur. In this report we present evidence that such a separate and distinct factor exists for the enzyme β-glucuronidase.

The structure of this enzyme in inbred strains of the house mouse, *Mus musculus*, is specified by a single gene on linkage group XVII.[1, 2] The enzyme resides within both the lysosomes and the membranes of the ergastoplasm in liver cells, and enzyme molecules resident at the two sites are physically indistinguishable and coded for by the same gene.[3] A mutation of this gene, originally discovered as a marked reduction of β-glucuronidase activity in all tissues,[4] also results in a change in the thermal stability of the enzyme.[3] Mutation of the structural gene significantly alters the proportion of the activity at the two sites.[3] Not surprisingly then, the structure of a protein apparently can affect its own intracellular distribution.

The results of the present study describe a new gene in the house mouse, distinct from the glucuronidase structural gene, which determines whether or not glucuronidase can be integrated into the ergastoplasmic membrane, thus demonstrating the existence of additional enzyme localizing factors. A preliminary report of several of these experiments has appeared previously.[5]

Materials and Methods.—Animals: DBA/2, C3H/HeHa, and YBR mice 90–120 days of age were all generously supplied from the breeding colony of Dr. T. S. Hauschka.

Preparation of tissue homogenates: Homogenates (5%) were prepared in cold 0.25 *M* sucrose, buffered at pH 7.4 with 0.02 *M* imidazole-HCl, using a Potter Elvehjem-type homogenizer.

Osmotic shock: Homogenates were centrifuged at 105,000 *g* for 30 min to separate particulate from soluble enzymes. The sediment was resuspended in hypotonic buffer (0.02 *M* imidazole-HCl, pH 7.4) in order to solubilize lysosomal enzymes. Solubilized enzyme was then separated from enzyme resistant to osmotic shock by centrifuging the resuspended particles at 105,000 *g* for 30 min. Enzyme activities of the first supernatant and the final supernatant and sediment were determined.

Differential centrifugation: The schedule of deDuve *et al.*[6] was followed using a Spinco no. 40 rotor. Four particulate fractions were separated in order of decreasing size: nuclear, mitochondrial, lysosomal, and microsomal, plus the final supernatant. For some experiments a combined cytoplasmic particle fraction was prepared by centrifuging at 105,000 *g* for 30 min after removal of the nuclear fraction.

Zone centrifugation: Three milliliters of the resuspended cytoplasmic particle fraction were layed over a nonlinear, 31-ml gradient of sucrose (see Fig. 1), ranging in concentration from 0.4 *M* to 2.1 *M* and buffered at pH 7.5 with 0.02 *M* glycylglycine-NaOH. The gradient was prepared according to the suggestions of Bock and Ling.[7]

Electrophoresis of microsomal protein: Sedimented microsomes were resuspended in 0.02 *M* imidazole-HCl buffer, pH 7.4, and the suspension was made 1% in deoxycholate. This mixture was allowed to stand at room temperature for 15 min and then centrifuged at 105,000 *g* for 60

min. Deoxycholate was removed from the supernatant fluid by dialysis against two 1000-ml changes of the imidazole-HCl buffer. The dialyzed extract of microsomal protein was then subjected to electrophoresis in polyacrylamide-gel columns according to the method of Davis[8] for serum protein. The protein preparation was separated at pH 9.5 using a constant current of 5 ma per gel during the 30-min run. The gels were then stained for protein with aniline blue-black, and unbound stain was removed electrophoretically. The stained gels were stored in 7% acetic acid and photographed.

Enzyme activity measurements: Glucuronidase (I.U.B. 3.2.1.31) was assayed at 56°C by a modification of the procedure of Nimmo-Smith[9] which employs the substrate p-nitrophenyl glucuronide (Cyclo Chemical Co.). The final reaction mixture contained enzyme, 0.001 *M* p-nitrophenyl glucuronide, 0.1 *M* acetate buffer pH 4.6, and 0.1% of the surfactant Triton X-100 (Rohm and Haas Co.). The latter was added to ensure complete activation of latent lysosomal enzymes.[10]

Glucose-6-phosphatase (I.U.B. 3.1.3.9) activity was determined at 30°C by measuring the amount of phosphorus[11] liberated by the enzymatic hydrolysis of glucose-6-phosphate. The final reaction mixture contained enzyme, 0.04 *M* glucose-6-phosphate (Sigma Chemical Co.), and 5×10^{-4} *M* EDTA, and was buffered with 3.5×10^{-3} *M* histidine (pH 6.5).

Further details: A more extensive description of these experimental procedures is contained in the report of a related study.[12]

Results.—During a search for genetic variants of β-glucuronidase, the YBR strain of inbred mice was found to have little, if any, glucuronidase activity in the ergastoplasm of liver cells.[12] This strain has been maintained in the past because it carries the A^y (yellow-lethal) mutation, the dominant allele of the *agouti* gene. A^y is lethal in the homozygous condition and causes yellow coat color and obesity when heterozygous. The allele is maintained by crossing the yellow heterozygotes ($A^y a$) with nonagouti (aa) animals. The genetic factor affecting glucuronidase was found to be independent of the state of the *agouti* locus, since all animals of this strain were affected. By preference, only the *nonagouti* segregants of the YBR strain were used in this study.

The upset in the intracellular distribution of glucuronidase was first detected by finding that all of the particulate glucuronidase of a YBR liver homogenate was released into solution after osmotic shock. In contrast, approximately 40 per cent of the particulate enzyme of most other strains remains particulate after osmotic shock.[12] The osmotic shock technique results in the solubilization of lysosomal enzyme, but not microsomal enzyme.[5] Since microsomes arise by fragmentation of the ergastoplasm at the time of homogenization, this result suggests a deficiency of ergastoplasmic enzyme in liver cells of YBR mice. Direct evidence for this was obtained using sucrose gradient fractionation.

When liver particles are centrifuged through an appropriate nonlinear sucrose gradient, two bands of particles rapidly separate. Lysosomes and microsomes have been identified in the lower and upper zones, respectively, on the basis of the distributions of marker enzymes, the relative content of these bands among the particulate fractions obtained after differential centrifugation, and their respective sensitivities to osmotic shock. These experiments are reported elsewhere.[12]

Using liver particles from normal mice, the resulting distribution of glucuronidase in the gradient is bimodal (Fig. 1). If, in YBR mice, microsomal glucuronidase is lacking, but lysosomal enzyme is retained, we should expect to find only a single, rapidly sedimenting peak when YBR particles are sedimented through a similar gradient. Figure 2 shows that glucuronidase activity was indeed lacking in the region of the microsomes and that only a single peak corresponding to the lysosomes

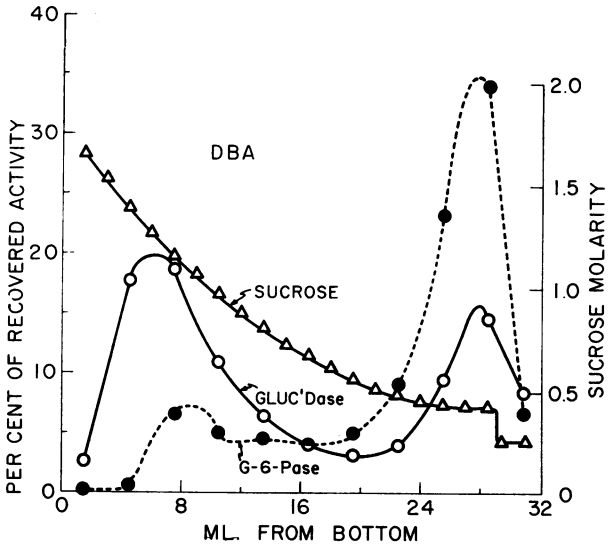

FIG. 1.—Distribution of enzyme after density gradient centrifugation of cytoplasmic particles from DBA/2 liver. The cytoplasmic particle fraction of a DBA/2 liver homogenate was layered over a nonlinear sucrose concentration gradient and sedimented at 24,000 *g* for 30 min. Fractions (3 ml each) were collected and assayed for glucuronidase (○—○) and glucose-6-phosphatase (●---●) activities. Activity in each fraction is expressed in terms of per cent of recovered activity as a function of volume previously removed from bottom of gradient. Sucrose concentration is designated by open triangles (△—△). Enzyme recoveries: glucuronidase, 14.3/15.8 activity units; glucose-6-phosphatase, 111/180 activity units.

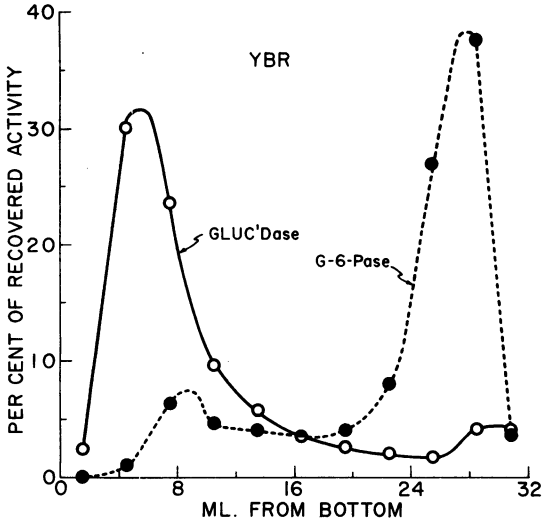

FIG. 2.—Distribution of enzymes after density gradient centrifugation of cytoplasmic particles from YBR liver. Procedures and symbols are identical to those of Fig. 1. Enzyme recoveries: glucuronidase, 4.2/4.8 activity units; glucose-6-phosphatase, 124/160 activity units.

TABLE 1

INTRACELLULAR DISTRIBUTION OF GLUCURONIDASE IN DBA AND YBR MOUSE LIVER

	Homog.	Nuc.	Mit.	Lys.	Mic.	Super.	Per cent recovery
Dry weight (% total)							
DBA (360 mg total)		14	22	6	20	37	99
YBR (374 mg total)		14	18	7	20	37	96
Total activity ($\times 10^3$)							
DBA	63.6	9.08	19.7	11.7	13.4	9.10	99
YBR	23.8	1.86	8.68	4.93	0.78	6.05	94
Specific activity							
DBA	177	648	895	1950	669	246	
YBR	63.7	133	483	705	39.2	164	
Specific activity ratio							
YBR/DBA	0.36	0.20	0.54	0.36	0.06	0.67	

Total activities are expressed as micromoles p-nitrophenol formed per hour at 56°C per gram of tissue. Specific activities are expressed as micromoles p-nitrophenol formed per hour at 56°C per mg dry weight. Relative specific activity is the activity per mg dry weight of any YBR fraction divided by that of the same DBA fraction. Dry weight refers to the TCA-insoluble material in each fraction.

was present. The distribution of the microsomal marker, glucose-6-phosphatase, was normal. The glucuronidase activity at the top of the gradient is soluble and presumably arose from damaged lysosomes.

Independent confirmation of this conclusion was obtained when liver homogenates of YBR and wild-type DBA/2 were fractionated by differential centrifugation, and the glucuronidase activity of each particulate fraction was determined. YBR activity levels in nuclei, mitochondria, lysosomes, and supernatant paralleled those of DBA, whereas the microsomal fraction of YBR had a substantially lower activity (Table 1).

Thus, three different criteria (osmotic shock, gradient centrifugation, and bulk fractionation) indicate that little, if any, glucuronidase is incorporated into the ergastoplasm of YBR liver cells. Moreover, the data reported in Table 1 suggest that the enzyme missing from the microsomes has not been displaced to another fraction.

The lack of glucuronidase in the ergastoplasm does not reflect a major upset in the accumulation of other proteins at this intracellular site, and several lines of evidence indicate that the anomaly is specific to glucuronidase. First of all, the microsomal fraction obtained by differential centrifugation of YBR liver homogenates contained normal amounts of trichloracetic acid-precipitable material (Table 1). Furthermore, the activity of glucose-6-phosphatase, another microsomal enzyme, was normal in YBR liver homogenates and was distributed like that of wild-type DBA/2 in a sucrose density gradient (Fig. 2). Finally, disk electrophoresis of protein extracted from YBR microsomes showed a pattern indistinguishable from that obtained with C3H mice and from F_1 progeny of the cross between these two strains (Fig. 3). All protein bands observed in the C3H electropherogram appear to be present in similar amounts in that of YBR and of F_1.

The conclusion that the anomaly is specific to glucuronidase and does not involve the bulk of the microsomal protein raises the question of whether this enzyme is no longer capable of being incorporated into the membranes of the ergastoplasm be-

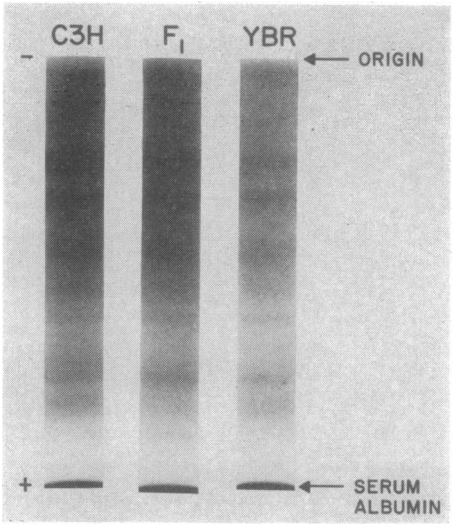

FIG. 3.—Disk electrophoresis of liver microsomal protein. Approximately 19 protein bands can be visually distinguished in the gels.

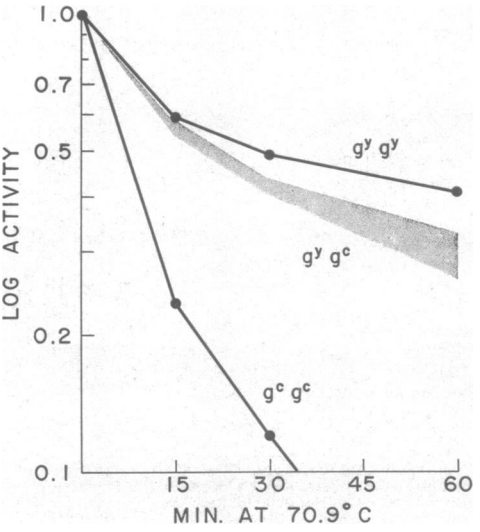

FIG. 4.—Heat inactivation of cell fractions. Survival of C3H(g^cg^c) and YBR (g^yg^y) forms of glucuronidase activity as a function of the duration of exposure to heating at 70.9°C. The shaded area represents the range of enzyme activity from each of the cellular fractions after inactivation. The fractions were derived from the the F_1 (g^yg^c) by the differential centrifugation scheme of deDuve *et al.*[6]

cause of a change in its structure. To answer this question, the YBR strain was crossed with the C3H/HeHa strain which possesses a form of glucuronidase distinguishable from that of the YBR by its rapid inactivation at 71°C.[3, 12] Both parental forms of the enzyme can be identified in the F_1 progeny of such a cross. If YBR enzyme cannot be inserted into the ergastoplasm, then only heat-labile glucuronidase activity should be found in the microsomal fraction of F_1 progeny.

Figure 4 shows the heat inactivation curves of each parental form of liver enzyme, as well as that of the enzyme activity present in each of the subcellular fractions obtained by the differential centrifugation of F_1 homogenates. The denaturation curves for all F_1 fractions fell within a narrow range, indicating that the relative proportions of the YBR and C3H types of enzyme molecules were similar in all fractions. Furthermore, as the position of the heat-denaturation curve shows, the preponderant form of the enzyme present in all fractions was that of the YBR parent. Thus, the enzyme present in the microsomes of the F_1 progeny is primarily of the YBR type, implying that a functional allele of some gene necessary for the insertion of the YBR form of the enzyme into the ergastoplasm has been restored by crossing with the C3H strain.

In order to decide whether the genetic factor missing in YBR was the C3H glucuronidase structural allele or another genetic element, the progeny of the backcross diagrammed in Figure 5 were examined. If the essential factor were the glucuronidase allele, only two classes of backcross animals would be expected: enzyme heterozygotes with microsomal enzyme and homozygotes with no micro-

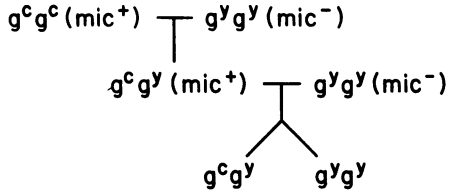

FIG. 5.—Backcross scheme. The symbol g^c refers to the mutant glucuronidase structural allele characteristic of C3H mice and g^y to that of YBR animals. The presence or absence of microsomal enzyme in each genetic class is indicated in parentheses.

somal enzyme. However, if the factor were distinct from the structural gene, then the recombinant classes should also occur. In order to determine which alleles were present at the enzyme structural locus in backcross progeny, the inactivation rate of spleen enzyme, rather than liver, was determined, since the C3H form of the enzyme is present in considerably greater amounts in spleen than in any other organ.[13] The presence or absence of microsomal enzyme in liver was determined by the osmotic shock technique rather than by differential centrifugation of cell particles. Because of the cross-contamination of fractions, differential centrifugation does not give an accurate measure of the absolute amount of enzyme present at each site.

The two techniques, inactivation of spleen enzyme and liver-particle osmotic shock, were combined to yield the results shown in Figure 6. The dashed lines represent the observed rates of denaturation of the spleen glucuronidase from mice of known genotypes. The shaded areas indicate the range of heat denaturation rates of spleen enzyme in backcross animals. Two distinct classes of inactivation curves are present corresponding to the two expected enzyme classes. The intracellular distribution of liver glucuronidase activity, tabulated on the right side of Figure 6, shows that each enzyme class included individuals with microsomal glucuronidase and individuals lacking microsomal glucuronidase. The existence of the g^yg^y (Mic+) class of backcross progeny shows that YBR-enzyme homozygotes

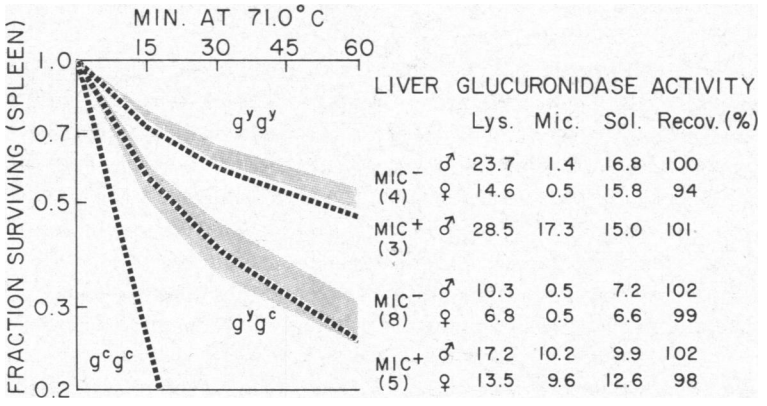

FIG. 6.—Recognition of backcross classes. Spleen enzyme survival is represented as a function of the duration of exposure to the enzyme to heat at 71.0°C. Dashed lines represent the observed rates of heat denaturation of spleen glucuronidase of mice with known genotypes. The shaded areas represent the ranges of heat-denaturation rates seen among backcross animals. Glucuronidase activities of the lysosomal, microsomal, and soluble liver fractions derived from 1 gm of liver of corresponding backcross animals are indicated on the right. The number of animals examined in each group is indicated in parentheses.

can acquire the ability to insert glucuronidase into microsomes as well as into lysosomes. The existence of the $g^y g^c$ (Mic$^-$) class indicates that this factor is equally required for the incorporation into the ergastoplasm of either structural form of the enzyme.

Thus, the genetic factor required for insertion of glucuronidase into the ergastoplasm assorts independently of the glucuronidase structural gene. The symbol E is proposed to represent this factor. The wild-type allele is symbolized as E^+, while the mutant allele, which results in an omission of glucuronidase from the ergastoplasm, is symbolized as e^{go}.

Since the E gene assorts independently of the previously mapped *glucuronidase* structural gene, linkage group XVII is eliminated as a probable location site. Linkage groups V and VIII are also excluded since the *agouti* and *brown* genes, segregating in the backcross progeny, assorted independently of E. Furthermore, an examination of the sex ratios and pedigrees of the backcross animals indicated that the gene is neither sex-linked nor maternally inherited.

In addition to the specificity of the E gene toward glucuronidase, preliminary evidence suggests that the effect of this gene is also tissue-specific since normal amounts of osmotically resistant glucuronidase activity (25–30% of total) are present in spleen homogenates.

Discussion.—The present study shows that a Mendelian factor, distinct from the glucuronidase structural gene, is required for the incorporation of β-glucuronidase into the membranes of the ergastoplasm. An altered form of this factor present in the YBR strain prevents the accumulation of glucuronidase in ergastoplasm of liver cells. The results suggest that the intracellular compartmentalization of enzymes may be controlled by factors other than the primary structure of the enzyme. To our knowledge, no other genetic factor with the same or analogous properties has been reported in multicellular organisms. However, Edgar and Wood[14] have demonstrated the existence of genes in phage T4D whose products are required for the morphogenesis of the phage particle.

Our results have additional significance when compared with the earlier report[3] describing a structurally altered form of glucuronidase whose relative distribution in the lysosomes and in the microsomes was changed. The interpretation favored in that case was that the altered enzyme protein possessed a different relative affinity for the binding sites of the lysosomes and the ergastoplasm. It now appears that in addition to the structure of glucuronidase itself, a separate form of nuclear control exists for the intracellular localization of liver glucuronidase.

The mechanism of action of this additional control cannot be determined from the results of the present study. However, two obvious possibilities present themselves for consideration: (1) the E^+ allele may code for an enzyme which acts upon native glucuronidase to produce an altered form of the enzyme which is now susceptible to integration in the ergastoplasmic membrane; or (2) the E^+ allele determines a factor essential to the construction of a proper glucuronidase binding site in the ergastoplasmic membrane. For the present we cannot choose between these alternatives. However, it should be noted that the E gene acts equally upon both structural forms of glucuronidase (Fig. 4).

During cell fractionation, the missing ergastoplasmic enzyme did not appear elsewhere in the cell. Furthermore, the residual lysosomal activity of YBR fell

within the range of lysosomal activities observed among normal inbred strains of mice.[12] These facts suggest that either the liver has a feedback system which limits the production of glucuronidase to the number of available intracellular sites, or that the cells have an efficient method of disposing of excess soluble enzyme.

An important feature of the behavior of the *E* gene is its apparent specificity toward glucuronidase. No gross upset in the accumulation of ergastoplasmic protein was found, as indicated by the presence of wild-type amounts of TCA-precipitable material and by the normal electropherogram of microsomal protein from YBR animals. Furthermore, the classical microsomal enzyme marker, glucose-6-phosphatase, was present in wild-type amounts and exhibited a typical microsomal localization after centrifugation of cell particles in a sucrose density gradient. Evidently, ergastoplasmic glucuronidase is under a nuclear control mechanism distinct from other proteins of this intracellular structure. If this specificity should prove to reside in special ergastoplasmic binding sites, the implication would be that most, if not all, ergastoplasmic proteins possess special binding sites.

We would like to infer that glucuronidase, and perhaps many other proteins, are under the control of a special class of genetic elements which determine whether a protein can exist in a functional state at a particular intracellular location.

Summary.—Liver cells of YBR mice lack ergastoplasmic glucuronidase, but retain the enzyme in lysosomes. The loss of ergastoplasmic enzyme is due to mutation of a Mendelian gene which is distinct from the glucuronidase structural gene. The new gene, symbolized as *E*, does not appear to affect other ergastoplasmic proteins, and is tissue-specific. The results demonstrate that the intracellular location of an enzyme can be determined by factors other than the primary structure of the enzyme, and that separate genes control these factors.

Note added in proof: We apologize for having unwittingly omitted reference to the recent and elegant paper of D. O. Woodward and K. D. Munkres (these PROCEEDINGS, **55**, 872 (1966) describing non-chromosomal genetic variants of mitochondrial structural protein. The present relevance of their work is quite obvious.

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[1] Paigen, K., and W. K. Noell, *Nature*, **190**, 148 (1961).

[2] Sidman, R. L., and M. C. Green, *J. Hered.*, **56**, 23 (1965).

[3] Paigen, K., *Exptl. Cell Res.*, **25**, 286 (1961).

[4] Morrow, A. G., E. M. Greenspan, and D. M. Carroll, *J. Natl. Cancer Inst.*, **10**, 657 (1949).

[5] Paigen, K., and R. Ganschow, in *Brookhaven Symposia in Biology*, no. 18 (1965), p. 99.

[6] deDuve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans, *Biochem. J.*, **60**, 604 (1955).

[7] Bock, R. M., and N. S. Ling, *Anal. Chem.*, **26**, 1543 (1954).

[8] Davis, B. J., *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).

[9] Nimmo-Smith, R. H., *Biochim. Biophys. Acta*, **50**, 166 (1961).

[10] Wattiaux, R., and C. deDuve, *Biochem. J.*, **63**, 606 (1956).

[11] Sumner, J. B., *Science*, **100**, 413 (1944).

[12] Ganschow, R., and K. Paigen, *Genetics*, in press.

[13] Paigen, K., these PROCEEDINGS, **47**, 1941 (1961).

[14] Edgar, R. S., and W. B. Wood, these PROCEEDINGS, **55**, 498 (1966).