Table 1. DEAE-cellulose chromatography of mammary-tissue culture fluid.

NaCl* (*M*)	Recovered † (%)	Labeled γA
0	2	0
0.1	18	0
0.2	24	+++
0.3	32	+
0.5	19	0
1.0	8	0
Not recovered	7	

* In 0.005*M* phosphate buffer, *p*H 7.9. † Protein precipitated with 5 percent trichloroacetic acid. Results expressed as fraction of original TCA-insoluble radioactive protein.

trichloroacetic acid (TCA) of the original culture fluid. To this pool was added as a carrier 15 mg of nonlabeled rabbit immunoglobulin G (γG); the mixture was extensively reduced and alkylated and passed through a previously calibrated Sephadex G-200 column in the presence of guanidine. The fractions containing heavy (α and γ) and light chains had about 40 and 60 percent, respectively, of the radioactivity applied. They were dialyzed, lyophilized, and analyzed by disk electrophoresis in the presence of urea. The procedures for separation and analysis of the polypeptide chains have been described previously (*4*). The resulting patterns were typical of γG heavy and light chains. The concentration of γA was insufficient to give visible α- or T-chain bands in these patterns. After dialysis against distilled water, the stained disk gels were laid flat on a lantern slide and embedded in 2 percent agarose in saline. The embedded gels were dried under wet filter paper and exposed for 8 weeks to Kodak Royal Pan film. Spots of very dilute C^{14}-lysine were placed near the ends of the gels to serve as markers for aligning the autoradiograph.

Figure 1 shows an electrophoretic pattern of the light chains with the corresponding autoradiograph. We found a radioactive band migrating more rapidly than the light chains, with a faint trail extending into the light-chain region. Densitometric analysis of the autoradiograph showed that 75 percent of the label in the gel was in the fast component in the area where T-chain migrates. In similar analysis of the heavy-chain fractions, most of the radioactive material was retained in the spacer gel and hence seemed to be contaminating large protein.

Our data show that T-chains, which are intimately associated with colostral γA, are synthesized by mammary tissue. This observation is consistent with that of Tomasi *et al.* (*1*) who reported that T-chains are present in parotid gland acinar cells; it also extends the finding of Hochwald *et al.* (*7*) who showed that human mammary and parotid gland synthesized a protein that coprecipitated with γA. Furthermore, it seems likely that the heavy and light chains of the colostral γA are synthesized elsewhere, in that there was no significant amount of label associated with them. This latter conclusion could be invalidated if the T-chain was particularly rich in lysine or isoleucine, or both, or if there were very large pools to dilute newly synthesized heavy and light chains. Both of these possibilities seem unlikely to us. Since at least three times as much radioactivity is present in T-chains as in light chains, and since there are probably twice as many light chains as T-chains in the whole molecule, a sixfold difference in content of the two amino acids would be required to explain our data. This is not consistent either with the observed electrophoretic mobility of T-chain or with the published amino acid composition of the whole molecule (*6*). To our knowledge, there is no evidence that large pools of intracellular γA exist in secretory glands. Immunofluorescent studies of human parotid tissue (*1*) indicate that γA is formed only in plasma cells which are probably actively synthesizing the protein.

Our experiments indicate that colostrum rich in γA can be produced without local synthesis of either light or heavy chains. Therefore, it seems likely that γA is actively transported and concentrated from serum into colostrum.

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References and Notes

1. T. B. Tomasi, E. M. Tan, A. Soloman, R. A. Prendergast, *J. Exp. Med.* **121**, 101 (1965).
2. M. A. South, M. D. Cooper, F. A. Wollheim, R. Hong, R. A. Good, *ibid.* **123**, 615 (1966).
3. R. Hong, B. Pollara, R. A. Good, *Proc. Nat. Acad. Sci. U.S.* **56**, 602 (1966).
4. J. J. Cebra and P. A. Small, Jr., *Biochemistry* **6**, 503 (1967); R. A. Reisfeld and P. A. Small, Jr., *Science* **152**, 1253 (1966).
5. G. M. Hochwald, G. J. Thorbecke, R. Asofsky, *J. Exp. Med.* **114**, 459 (1961).
6. J. J. Cebra and J. B. Robbins, *J. Immunol.* **97**, 12 (1966).
7. G. M. Hochwald, E. B. Jacobson, G. J. Thorbecke, *Fed. Proc.* **23**, 557 (1964).
8. Goat antiserum specific for γA was provided by Dr. J. J. Cebra.

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Ultrastructure of Gamma M Immunoglobulin and Alpha Macroglobulin: Electron-Microscopic Study

Abstract. *Electron microscopy of purified Waldenström macroglobulins and normal human and rabbit γM immunoglobulins revealed spider-like structures with five legs varying in length and often joining a central ring. Usually only the central more rigid part of this structure (about 150 by 170 angstroms) was clearly visible, but occasionally particles were seen with longer very flexible legs having a total span of about 350 angstroms. Molecules of γM antibody retained antibody activity during preparation for electron microscopy. Human and rabbit alpha macroglobulins revealed more rigid symmetric structures (100 by 200 angstroms) which resembled the Russian letter* ж.

The size and conformation of several serum proteins have been estimated by light scattering, x-ray diffraction, fluorescence polarization, intrinsic viscosity measurement, analytical ultracentrifugation, and electron microscopy. Some of these techniques have been applied to studies of myeloma proteins and Waldenström macroglobulins. But we are aware of no reported electron-microscopic studies of these paraproteins, and the only electron-microscopic data relating to normal γM immunoglobulins, not in complexes with antigen, have been published by Höglund and Levin (*1*) and Höglund (*2*). The limited application of electron microscopy in studies of serum macroglobulins can be partially ascribed to their apparent low electron density.

A different situation exists when the γM globulins have antibody activity and can be examined when they form complexes with an antigen of well-defined and characteristic shape. This approach has recently been utilized in electron-microscopic studies of complexes of virus and γM antibody (*2–4*). In

one of these studies (*4*), the biological activity of the γM antibody was correlated to its capacity to form visible complexes with the antigen. These results led us to attempt an electron-microscope study of purified γM globulins that were not complexed with antigen. We now report observations on the ultrastructure of the following proteins: rabbit γM immunoglobulins with antibody activity to poliovirus, normal human γM globulins, Waldenström macroglobulins, and—for comparison—human alpha macroglobulins (α_2M) and rabbit alpha macroglobulins (α_1M).

The γM globulins examined were from several sources. The two rabbit γM preparations and two normal human γM preparations were purified in our laboratory by methods outlined below; three Waldenström macroglobulin preparations were supplied by Drs. H. Metzger and R. Norberg, purified as described (*5*).

Six αM globulin preparations were studied. Two human α_2M and two rabbit α_1M preparations were purified in this laboratory; an additional human α_2M preparation was supplied by Dr. P. O. Ganrot and was purified as described (*6*); the final human α_2M preparation was supplied by Dr. G. Schwick, Behringwerke AG.

The rabbit and human γM and αM globulins purified in this laboratory were prepared by a five-step procedure including: (i) removal of low-density lipoproteins by precipitation with dextran sulphate (molecular weight, 560000) and Ca^{2+} (*7*), (ii) precipitation of the macroglobulins with 7.5 percent (weight/volume) polyethylene glycol (molecular weight, 6000) (*8*), (iii) subjecting the redissolved macroglobulins to gel filtration on Sephadex G-200 or Sepharose 4B, (iv) fractionation of the first eluted protein peak in a sucrose-density gradient, and finally (v) separation of the obtained γM and αM globulins by zone electrophoresis on polyvinyl chloride (PVC) (*9*). No lipoproteins were detectable by double diffusion in 1 percent agar gel (LKB immunodiffusion equipment) in the macroglobulin preparations subjected to electrophoresis.

In order to prepare rabbit γM globulins with antibody activity to poliovirus, 4- to 5-month-old rabbits, weighing 2 to 3 kg, were injected intravenously with 3 ml [4×10^8 plaque-forming units (PFU) per milliliter] of poliovirus, type 1, produced and purified on a CsCl gradient (*4*). The rabbits were bled by cardiac puncture 10 days after immunization, the blood was allowed to clot, and the serums were stored at −20°C without preservatives. Such serums or γM globulin preparations were titrated for virus-neutralizing antibodies by mixing equal volumes of virus (150 PFU) and varying dilutions of the serum or γM preparation. The mixtures were held for 20 hours at 3°C and then 4 hours at room temperature, and assayed without dilution on HeLa cell monolayers by the plaque-inhibition method.

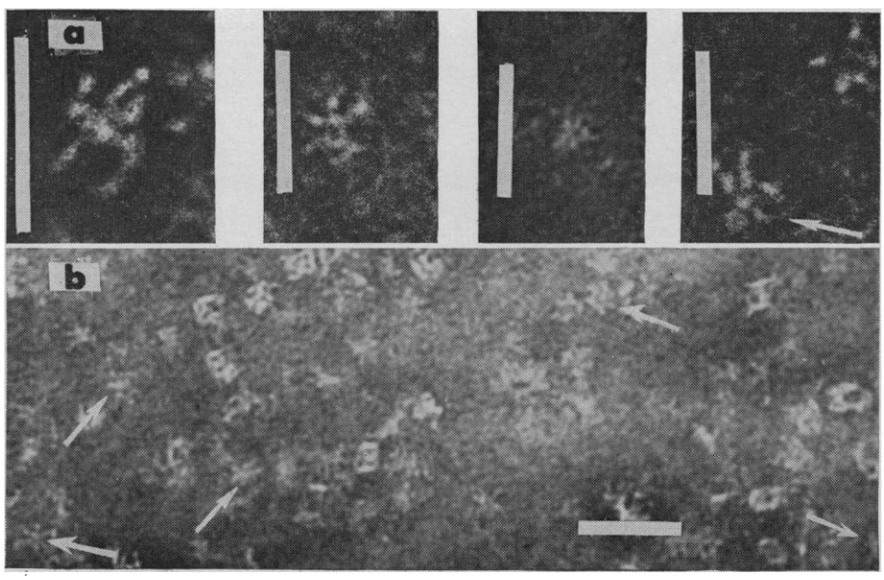

Fig. 1. Electron micrographs of purified rabbit and human γM globulins. (a) Spider-like structures with five legs of varying length in both normal and pathological γM globulin preparations. (b) Normal human γM preparation showing both the spider-like structures (arrows) and loop formation apparently due to folding. The scale represents 60 mμ.

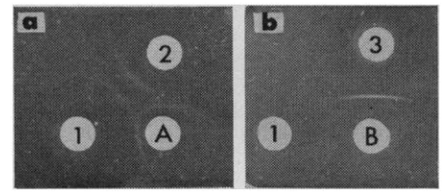

Fig. 2. Agar double-diffusion analysis of (a) normal human γM globulin and (b) human α_2M globulin. Well *A*: purified normal human γM globulin; well *B*: purified human α_2M globulin; wells 1, 2, and 3: antiserum to human serum, rabbit antiserum to human γM globulin and rabbit antiserum to human α_2M globulin, respectively. The precipitation lines between wells 1 and 2 are due to the reaction of the horse antiserum to human serum (well 1) with human serum proteins added to the rabbit serum (well 2) when removing rabbit antibodies not directed against human γM globulin.

Specimens for electron microscopy were prepared by placing a drop of a suspension of macroglobulin and 2 percent sodium tungstosilicate on a 400-mesh carbon-coated grid; the excess fluid was removed with a filter paper after a few seconds. The grids were immediately transferred into the vacuum compartment of a JEM-5Y electron microscope and examined at an initial magnification of 50,000; double condensed illumination was used.

Initial electron micrographs of isolated complexes of poliovirus and rabbit γM antibody (*4*) occasionally revealed, in addition to the complexes, structures—not associated with virus—having principally two different shapes, one of uniform size resembling the Russian letter ж, and the other a structure resembling a spider with five legs of varying lengths (modifications of this latter structure showed loop formation). Since at this stage of the study the final electrophoresis step was omitted in the purification procedure, the macroglobulin preparation with which the virus was incubated contained both γM and αM globulins. Thus, it was impossible to determine which, if either, of the free-lying structures actually represented the γM antibody molecules. However, when rabbit γM and αM globulins were separated by zone electrophoresis, evidence pointing to the identity of the spider-like structures with γM globulin molecules was obtained. Electron micrographs of fractions, within the γM region of PVC blocks containing the peak of the antibody activity revealed a high concentration of flexible spider-

like structures with five legs often joining a central ring and occasionally occurring in small aggregates (Fig. 1a). An often-observed modification of the latter structure involved the folding of the legs in toward the central midpoint, thus forming double loops on one or both sides (Fig. 1b). In a previous study of complexes of poliovirus and rabbit γM antibody (*4*), such double loops were often seen attached to the virus capsid.

Fractions from the γM region of the PVC blocks had poliovirus neutralizing-antibody activity in the plaque-inhibition neutralization test. In addition, these γM antibodies formed specific complexes with poliovirus which were visible in the electron microscope, an indication that the γM antibodies retained antigen-binding capacity during both purification and preparation for electron microscopy. In the complex, the antigen may have a stabilizing effect on the combining site of the antibody molecule, and therefore we tried to ascertain whether purified, γM antibody molecules not associated with antigen retained activity during the staining and drying necessary for electron microscopy. These experiments indicated that the γM antibody preparations retained at least 50 percent of their original antibody activity when redissolved in phosphate-buffered physiological saline after this treatment (*9a*).

The human γM preparations (0.5 to 0.6 mg of protein per milliliter) which gave a single line of precipitation when tested against horse antiserum to human serum and horse or rabbit antiserum to human γM globulin by the Ouchterlony technique (*10*) (Fig. 2a), revealed structures indistinguishable from those seen in the rabbit γM preparations.

Three different γM macroglobulin preparations (type Waldenström), containing 5 to 15 mg of protein per milliliter, were examined in the electron microscope. All three preparations showed the same general features: five-pointed spider-like particles occasionally entangled in small aggregates were the predominating structures (Fig. 3). There was great variability in the lengths of the legs of these particles, and thus the overall dimensions varied between 150 $\times$ 170 Å for the denser, more compact structures to 150 $\times$ 350 Å in the case of particles whose legs appeared maximally extended.

A single line of precipitation was obtained when two of the Waldenström γM preparations were tested by immunoelectrophoresis against horse antiserum to human serum and rabbit antiserum to human γM globulin by the micromethod of Scheidegger (*11*).

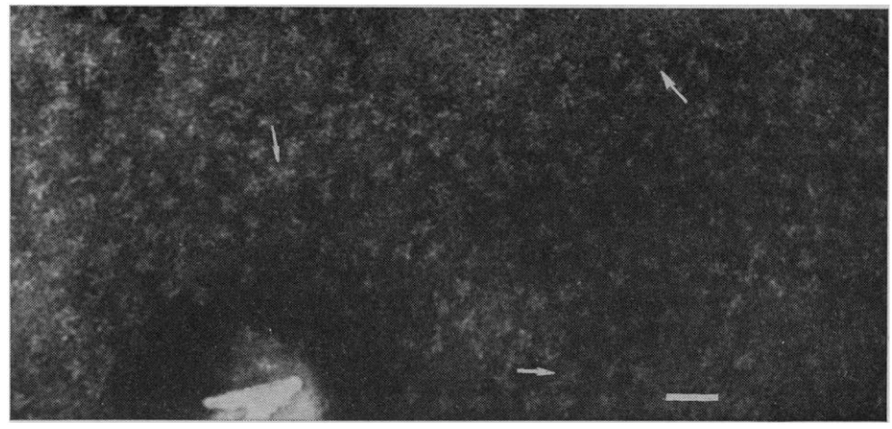

Fig. 3. Electron micrograph of human γM globulin (type Waldenström) revealing a high concentration of flexible spider-like structures with five legs, occasionally occurring in small aggregates. Usually only the central, more rigid, electron-opaque part of the structure is clearly visible (see arrows). The scale line represents 50 mμ.

Electron micrographs of purified human α_2M and rabbit α_1M globulin revealed a high concentration of intact structures resembling the Russian letter ж. These structures were more symmetric and rigid than the spider-like particles observed in purified γM preparations and seemed to have shorter side legs (Fig. 4). The modal values for the breadth (the horizontal bar) and height of apparently intact α_2M molecules were estimated to be 90 to 100 Å and 180 to 200 Å, respectively. Besides apparently intact αM particles, partially collapsed particles were occasionally seen in some areas of the grid which were covered with a rather thick layer of the STS stain. The rabbit α_1M and human α_2M preparations were analyzed by immunoelectrophoresis and double-diffusion in agar, with sheep antiserum to rabbit serum, horse antiserum to human serum and rabbit antiserum to human α_2M globulin. These tests revealed a single precipitation line typical for αM in each system (Fig. 2b).

Much of our knowledge of immunoglobulins has emerged from studies of myeloma proteins and Waldenström macroglobulins. The results have subsequently been confirmed in studies of normal immunoglobulins and specific antibodies. But it is still an open question whether the paraproteins are truly abnormal serum proteins or merely extreme elevations of certain regions within the normal γ-globulin moiety.

In our study no distinct difference in the electron-microscopic appearance of normal γM globulins and Waldenström macroglobulins was observed. The spider-like structures were dominant in both kinds of preparations, while some of the electron micrographs suggested that normal γM preparations contained a greater number of apparently intact such structures with long, thin, very flexible legs. This can, however, be a spurious finding since the purified normal γM preparations examined were fresher than the Waldenström macroglobulins. In addition, the two types of

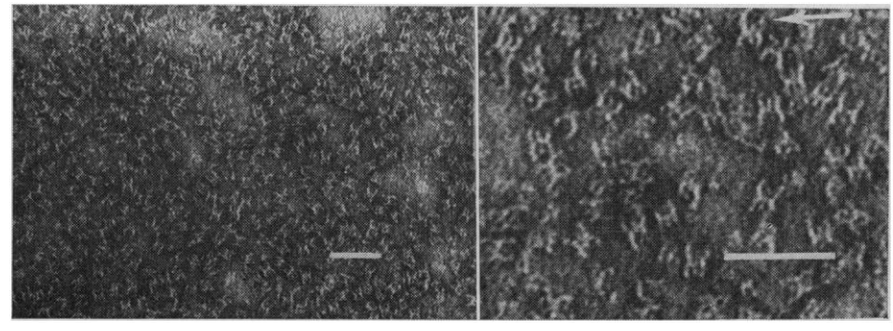

Fig. 4. Electron micrographs of purified human α_2M globulin. Structures resembling the Russian letter ж are seen in high concentration (see arrow). The scale lines represent 50 mμ.

γM preparations were purified by different methods.

Although it is not unreasonable that the γM molecule might have an ultrastructure somewhat resembling that of the αM globulin, since many of their physicochemical properties indicate a strong similarity, we consider it unlikely that the spider-like particles observed in the γM preparations represented a contamination with αM globulin molecules for the following reasons. (i) There were recognizable differences in the shape of these two structures, the spider-like structures in the γM preparations being more slender and less symmetric with longer, more flexible side legs than the αM particles. (ii) No contamination with αM globulins was detectable in the γM preparations by the Ouchterlony technique or immunoelectrophoresis. The maximum contamination that could escape detection was about 12 to 15 μg/ml, which would amount to about 2 percent of the total protein in the γM preparations. Therefore, the number of αM molecules expected in the preparations would be far less than the observed number of spider-like particles. (iii) In the γM preparations, structures could be seen at various stages of transition from apparently intact extended spider-like particles to those whose legs folded together to form small loops, which were remarkably similar to the loops surrounding the poliovirus capsids in the poliovirus-γM complexes (*4*).

In Höglund and Levin's (*1*) and Höglund's (*2*, *12a*) electron-microscopic studies of normal human γM globulin and γM antibodies against T2 bacteriophage, the γM molecule was reported to have an ovoid shape with dimensions of about 200 × 300 Å. This large ellipsoid structure is not in accordance with our observations on both pathological and normal human γM globulins. In addition, Rowe (14) has pointed out that the molecular weight, calculated on the basis of Höglund and Levin's dimensions differs grossly from values generally accepted for γM.

Humphrey and Dourmashkin (*13*) and Feinstein and Munn (*14*) have published electron micrographs showing filamentous structures, considered to represent γM antibodies, attached to the erythrocyte membrane and bacterial flagella, respectively. The length of these structures, which appears to be about 100 to 150 Å, is compatible with the size of the isolated folded γM molecules that we examined. The electron microscopic appearance of complexes of virus and γM antibody has been studied in detail (*3*, *4*). The flexibility of the γM molecule and the tendency to react with the virus with the formation of two to four loops on the surface of the virus capsid are consistent with our present results. The structure here considered to represent the γM molecule is also compatible with the suggestion (*15*) that the molecule should have five 6*S* to 7*S* subunits (Fig. 1a).

That γM globulins apparently yield intrinsic viscosity values which are higher than those typical for "globular" proteins (*16*) has been attributed to a structure that is either a rigid asymmetric ellipsoid or one that has a high degree of intramolecular, rotational freedom. The latter explanation is compatible both with data from measurements of fluorescence depolarization (*17*) and with the observation that the γM subunits are probably linked by only two disulfide bonds per subunit (*18*). This concept of great intramolecular flexibility was supported by our ultrastructural study.

Our data are also compatible with earlier data on fluorescence depolarization (*19*), an indication that the α_2M molecule is more rigid than the γM molecule. The ellipsoidal or spherical structure proposed earlier (*1*, *20*) for the α_2M globulin is difficult to reconcile with the electron-microscopic appearance of this molecule in our study.

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References and Notes

1. S. Höglund and Ö. Levin, *J. Mol. Biol.* **12**, 866 (1965).
2. S. Höglund, *Int. Congr. Electron Microscopy, 6th, Kyoto, Abstr.*, p. 123 (1966).
3. J. D. Almeida, F. Brown, A. P. Waterson, *J. Immunol.* **98**, 186 (1967).
4. S-E. Svehag, and B. Bloth, *Virology* **31**, 676 (1967).
5. F. Miller and H. Metzger, *J. Biol. Chem.* **240**, 3325 (1965).
6. P. O. Ganrot and B. Scherstén, *Clin. Chim. Acta* **15**, 113 (1967).
7. M. Burstein and J. Samaille, *ibid.* **3**, 320 (1958).
8. P. Å. Albertsson, *Partition of Cell Particles and Macromolecules* (Wiley, New York, 1960), p. 154.
9. H. J. Müller-Eberhard, H. G. Kunkel, E. C. Franklin, *Proc. Soc. Exp. Biol. Med.* **93**, 146 (1956).
9a. B. Chesebro, B. Bloth, S-E. Svehag, in preparation.
10. Ö Ouchterlony, *Acta Path. Microbiol. Scand.* **25**, 186 (1948).
11. J. J. Scheidegger, *Int. Arch. Allergy Appl. Immunol.* **7**, 103 (1955).
12. J. Rowe, *J. Mol. Biol.* **16**, 553 (1966).
12a. S. Höglund, *Virology* **32**, 622 (1967).
13. J. H. Humphrey and R. R. Dourmashkin, in *Complement*, G. E. W. Wolstenholme and J. Knight, Eds. (Churchill, London, 1965), p. 175.
14. A. Feinstein and E. A. Munn, *J. Physiol.* **186**, 64 (1966).
15. F. Miller and H. Metzger, *J. Biol. Chem.* **241**, 1732 (1966).
16. V. K. Jahnke, W. Scholtan, F. Heinzler, *Helv. Med. Acta* **25**, 2 (1958); F. Miller and H. Metzger, *J. Biol. Chem.* **240**, 3325 (1965).
17. C. Georges, S. Guinand, B. Arrio, *Compt. Rend.* **261**, 3687 (1965); H. Metzger, R. L. Perlman, H. Edelhoch, *J. Biol. Chem.* **241**, 1741 (1966).
18. F. Miller and H. Metzger, *J. Biol. Chem.* **240**, 4740 (1965).
19. Y. Jacquot Armand and S. Guinand, *Biochim. Biophys. Acta* **133**, 289 (1967).
20. M. Schönenberger, R. Schmidtberger, H. E. Schultze, *Z. Naturforsch.* **13b**, 761 (1958).
21. Supported in part by the Swedish Medical Research Council (Project No. K 66-826 and B 67-16X-744-02B) and by the foundations "Konung Gustaf V:s 80-årsfond" and "Therese and Johan Anderssons Minne". One of us (B.C.) received a research stipend from Harvard Medical School. We thank Mrs. B. Andersson and Mrs. A. Hanko for assistance and Drs. H. Metzger, R. Norberg, G. Schwick, and P. O. Ganrot for macroglobulins.

1 June 1967

Enzyme Concentrations in Tissues

Abstract. *Apparent enzyme concentrations in cells and in mitochondria are calculated from data available in the literature. These values are 10^{-6} to 10^{-5} moles per kilogram of tissue. It is pointed out that these concentrations are much higher than those used in enzymatic studies in vitro. Metabolic interpretations of experiments in vitro should consider this additional departure from conditions in vivo.*

Experiments with purified enzymes for the study of metabolic control attempt to use physiological concentrations of substrates and effectors. Because in most cases sensitive assays are usually available for such studies, enzyme concentrations of about 10^{-7} to 10^{-10} mole/liter are used. In addition to limitations of technique and insufficient quantities of pure enzyme, a lack of knowledge of cellular enzyme concentrations has prevented adequate study of the effects of enzyme concentrations on proposed metabolic regulatory interactions in vivo.

Quite different kinetic behavior results when high concentrations of enzymes (10^{-5} to 10^{-6} mole/liter) are used instead of assay concentrations (10^{-7} to 10^{-10} mole/liter). I have known (*1*) that $10^{-5}M$ palmityl coenzyme A, a proposed regulator for citrate synthase, is effective against $10^{-8}M$ enzyme but not inhibitory for $10^{-6}M$ enzyme. Frieden and Colman (*2*) showed that $10^{-6}M$ glutamate dehydrogenase ex-