Isolation and Characterization of Glutamine Synthetase from Chicken Neural Retina

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SUMMARY

A procedure is described for the isolation of glutamine synthetase (EC 6.3.1.2) from chicken neural retina, and the enzyme has been purified to homogeneity. Its amino acid composition is given and the results of electron microscopic examinations are described. Electrophoresis in polyacrylamide gels containing either sodium dodecyl sulfate or urea showed that the enzyme consists of homogeneous subunits with a molecular weight of 42,000 ± 2,000. The molecular weight of the native enzyme was found by sedimentation equilibrium analysis to be 392,000. The sedimentation coefficient of the enzyme (15.8 S) and the Stokes radius (59 Å) were determined and their combination gave a molecular weight of 396,000. Calculations from these data would indicate the presence of nine subunits in the native enzyme; however, other considerations favor an octameric structure with a molecular weight of 340,000. The possible reasons for the apparent discrepancy between this value and those obtained from the sedimentation data are discussed. Electron microscopy showed the native enzyme molecules as rectangular particles with dimensions of about 90 x 90 x 125 Å; occasionally the individual molecules were seen associated in chain-like aggregates.

Glutamine synthetase (L-glutamate ammonia ligase (EC 6.3.1.2)) catalyzes the reaction L-glutamate + NH₃ + ATP → glutamine + ADP + Pi. Meister and his coworkers isolated glutamine synthetase from Escherichia coli (6) and rat liver (8) have also been examined. Although there are considerable similarities between these two bacterial enzymes, none of these are physically or chemically identical. It has long been known that in the neural retina of the chicken the level of glutamine synthetase is very high, and that its specific activity there is considerably above that in other tissues, including the brain (9). The discovery of the hormonal induction of this enzyme in the embryonic neural retina of the chick in vivo and in vitro by dexamethasone (10) has focused considerable interest on this system as a particularly favorable model for analysis of control mechanisms in embryonic differentiation (11). The induction of glutamine synthetase in the retina represents de novo synthesis and accumulation of the enzyme (12-14); it is correlated with the functional differentiation and maturation of the retina, and considerable information has been forthcoming concerning macromolecular events and gene expression in this system (9-14). A detailed knowledge of the properties of the enzyme is essential for further analysis of this enzyme induction, as well as for comparative studies of glutamine synthetases from various microbial and eukaryotic systems. We describe here the isolation of glutamine synthetase from chicken neural retina tissue and some physicochemical properties of the enzyme.

MATERIALS AND METHODS

Materials—Eyes from freshly slaughtered fowl were used as the source of retina tissue. The retina was removed from the eyes, thoroughly washed with cold Tyrode's physiological salt solution and lyophilized. About 2 g of lyophilized retina could be obtained from 200 heads. The sources of the chemicals were as follows: DEAE-cellulose, 0.93 meq per g (Sigma); hydroxyapatite for chromatography (Bio-Rad); catalase (Nutritional Biochemicals); and ferritin from horse spleen (Mann).

Assays—Glutamine synthetase activity was assayed by the hydroxamate reaction, as described before (11). However, for the determination of enzyme activity in the hydroxyapatite column fractions, this method could not be used because of interference due to high phosphate concentration. For this, 50 µl of each fraction was diluted to 0.35 ml with 0.1 M acetate buffer, pH 5, prior to reaction with hydroxylamine. Catalase was assayed by following the decrease in absorbance at 240 nm of a 3-ml reaction mixture containing 30 µmoles of potassium phosphate buffer, pH 7.2, 18 µmoles of hydrogen peroxide, and 25 µl of the sample (15). Activities were determined in terms of increase in absorbance at 240 nm/20 s/25 µl of enzyme fraction.
Ferritin was determined spectrophotometrically by measurement of absorbance at 415 nm.

**Protein Determination**—Protein concentrations were measured by the procedure of Lowry et al. (16) with crystalline bovine serum albumin as the standard.

**Disc Gel Electrophoresis**—SDS\(^{1}\)-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (17). For electrophoresis of the native, undissociated enzyme, 4% gels were used in 0.005 \( \text{M} \) phosphate buffer, pH 7.8. Electrophoresis in urea was performed after exposing the enzyme to 4 \% urea for 3 hours at 25°. The 4 \% of polyacrylamide gels used for this purpose also contained 4 \% urea in 0.005 \% phosphate buffer, pH 7.8. A current of 8 to 10 ma per tube was generally applied for 3 to 4 hours and after the run the gels were stained with Coomassie blue and destained electrophoretically.

**Zonal Centrifugation**—Sedimentation coefficients were measured by the method of Martin and Ames (15). Samples containing approximately 0.3 mg of each protein in a total volume of 0.1 ml were layered over 4.5 ml of 5 to 20 \% sucrose gradients in 0.05 \text{M} Tris-HCl buffer, pH 7.5, or in 0.01 \text{M} phosphate buffer, pH 7.4 (the latter was used when protein determinations by the Lowry procedure were desired). Centrifugation time was 18 hours at 35,000 rpm in Spinco SW 39 rotor at 4°. After piercing the tubes, the contents were fractionated and assayed for glutamine synthetase and marker protein peaks.

**Gel Filtration**—The gel filtration experiments employed a column of Sephadex G-200 (Pharmacia). In general, the procedure of Siegel and Monty (18) was followed. The buffer consisted of 0.1 \text{M} sodium phosphate, pH 8.0. After the column had been fully equilibrated with buffer, 1.35 ml of a solution containing 10 mg of serum albumin, 5 mg of catalase, 10 mg of ferritin, and 1.5 mg of retinal glutamine synthetase in 0.4 \text{M} phosphate buffer, pH 8, with 10 \% glycerol was applied to the column, and 1.4-ml fractions were collected. The peak positions in the eluate were determined and the parameter \( K_d \) defined by the following equation (18) was calculated.

\[
K_d = \frac{V_e - V_0}{V_t - V_e - V_0}
\]

where \( V_e \) is the elution volume at the peak, \( V_0 \) is the void volume of the column, \( V_t \) is the volume of the gel components, and \( V_t \) is the total volume of the gel bed.

**Analytical Ultracentrifugation**—A Spineco model E ultracentrifuge equipped with Raleigh interference optics was used. Sedimentation equilibrium measurements were made at three different concentrations of glutamine synthetase in the Yphantis six-channel centerpiece (19). Fringe displacements were measured with a two dimensional Nikon microcomparator. Sedimentation velocity measurements of the enzyme activity were made with a separation cell following the procedure of Yphantis and Waugh (20).

**Electron Microscopy**—Preparations of purified glutamine synthetase in 0.1 \text{M} phosphate buffer, pH 6.5, were applied to carbon-coated, 400 mesh copper grids, rinsed with 0.1 \text{M} potassium chloride, and negatively stained with one of the following solutions: 2 \% uranyl acetate, unbuffered; 1 \% sodium phosphotungstate, pH 7; or 1 \% ammonium molybdate, pH 7. In some experiments, negative staining was applied following fixation of specimens in 2 \% glutaraldehyde, pH 7. Comparable results were obtained with all three stains with or without prior fixation; since image contrast was best with uranyl acetate, this was employed in the present work, without prior fixation.

Since measurements of particle dimensions of glutamine synthetase, the enzyme preparations were negatively co-stained with one of two internal standards, tobacco mosaic virus\(^2\) or trypomycin-sin tactoids (21). Both of these macromolecules have well documented dimensions which have been established by x-ray diffraction and electron microscopy (21, 22). The outside diameter of tobacco mosaic virus is 180 \AA; the trypomycin-sin tactoids possess a transverse periodicity of 396 ± 6 \AA. Electron microscopy was performed with an AEI EM 6B instrument operating at 60 \text{kV}, with a 50 \text{m} objective aperture and utilizing original magnifications of 20,000 or 40,000 diameters.

**Amino Acid Composition**—Amino acid analyses were carried out after hydrolyzing the enzyme for 20 and 40 hours in a sealed hydrolysis tube in 6 \text{N} HCl. Portions of the hydrolysate were analyzed with a Beckman amino acid analyzer model 120C.

### RESULTS

#### Isolation of Retinal Glutamine Synthetase

The enzyme preparations were maintained between 0–4° throughout this procedure. All buffers contained 0.005 \text{M} mercaptoethanol. A small sample from each step was assayed for enzyme activity and protein content. A summary of the isolation procedure is shown in Table I.

**Table I**

| Step | Volume | Protein | Glutamine synthetase activity | Specific activity | Yield |
|------|--------|---------|-------------------------------|-------------------|-------|
|      | ml     | mg      | Total activity \( \text{units} \) | \( \text{per mg} \) | %     |
| 1. Crude extract | 40.5 | 2.84 | 115.0 | 418 | 16,524 | 148 | 100 |
| 2. Precipitate from AmSO\(_4\) fractionation | 4.0 | 4.21 | 16.84 | 2,155 | 8,620 | 512 | 52 |
| 3. Effluent from hydroxyapatite column | 5.0 | 0.41 | 2.05 | 410 | 2,050 | 1,000 | 12 |
| 4. Effluent from DEAE-cellulose column | 2.2 | 0.40 | 0.88 | 550 | 1,210 | 1,375 | 7 |

\(^1\) Taken from 400 mg of lyophilized chicken retina tissue.
\(^2\) GHA, micromoles of glutamylhydroxamate formed per hour per mg of protein.

### Table I

**Purification of glutamine synthetase from chicken retina**

1. Crude extract.
2. Precipitate from AmSO₄ fractionation.
3. Effluent from hydroxyapatite column.
4. Effluent from DEAE-cellulose column.

\( \text{Yield} \) is based on total protein and total activity of the crude extract.

### Notes

1. The tobacco mosaic virus was kindly provided by Professor Robert Haselkorn, Department of Biophysics, University of Chicago.
another 15 ml of saturated ammonium sulfate. The mixture was centrifuged after 15 min and the precipitate was dissolved in 3 to 4 ml of 0.005 M phosphate buffer and was dialyzed overnight against the same buffer.

Step 3. Chromatography on Hydroxylapatite Column—The dialyzed preparation from Step 2 was applied to a hydroxylapatite column (15 × 300 mm, bed height 15 cm). Potassium phosphate buffer (0.1 M; pH 7) was passed through the column at about 50 ml per hour with a peristaltic pump; 5 ml fractions were collected, and the absorbance of the fractions was read at 280 nm; when the absorbance of the eluent dropped to the level of the buffer (after about 150 ml of buffer), elution was begun with a gradient of 0.15 to 0.35 M phosphate buffer, pH 7.2. The enzyme emerged from the column between 0.2 to 0.3 M buffer concentration. All fractions with enzyme activity were pooled, and the combined eluent was concentrated to about 2 to 3 ml by passing through Amicon PM-30 membrane filters (43-mm diameter) in a 52-ml Amicon filtration cell under 50 to 50 ps.i. nitrogen pressure. These filters were tested before and were found to retain the enzyme almost quantitatively. The concentrate from the filtration cell was dialyzed overnight against 0.005 M phosphate buffer, pH 7.8, to remove the salts and then used in Step 4.

Step 4. Chromatography on DEAE-cellulose Column—The solution from Step 3 was applied to a DEAE-cellulose column (15 × 300 mm; bed height 15 cm) previously equilibrated with 0.005 M phosphate buffer, pH 7.8. About 150 ml of buffer was passed through the column at a flow rate of approximately 50 ml per hour. Under these conditions, glutamine synthetase remained tightly bound to DEAE-cellulose while some other proteins were washed out. The enzyme was then eluted with 0.1 M phosphate buffer of pH 6.5. Three-milliliter fractions were collected. The enzyme activity appeared within first 5 to 10 fractions. The active fractions were pooled and concentrated with PM 30 filters. The enzyme solution could be stored in ice in 0.1 M phosphate buffer, pH 6.5, for at least a month without any significant loss in activity.

Molecular Weight of Native Enzyme

The molecular weight of the native enzyme was determined by the sedimentation equilibrium method of Yphantis (19). Experiments were performed at three different concentrations (0.735, 0.55, and 0.75 mg per ml) in 0.1 M phosphate buffer, pH 6.5.

Plots of log ((Y - Y₀) + 3) versus r² were made and the slopes were determined by least squares fit, using those values which were clearly in the linear region, and neglecting the fringe displacements where they were densely packed. The mean molecular weight calculated from the three slopes (1,465, 1,477, and 1,539) was 392,000.

An independent estimate of the molecular weight of the native enzyme was obtained by combining the sedimentation coefficient and the Stokes radius according to the procedure of Siegel and Monty (18). This method, although less accurate than the sedimentation equilibrium method described earlier, allows measurements to be based on enzyme activity.

The sedimentation coefficient of retinal glutamine synthetase was found to be 15.8 ± 0.2 S from zonal centrifugations (15) with catalase and bovine serum albumin as standards. This value was in agreement with the s₁₀,₀ of 16.8 obtained from measurements made in the analytical ultracentrifuge with the separation cell and based on enzyme activity (20).

The Stokes radius of retinal glutamine synthetase was determined by chromatography on a column of Sephadex G-200 with bovine serum albumin, catalase, and ferritin as standards. From the plot of Kₚ versus the Stokes radius, a, for the marker proteins (Fig. 1), the Stokes radius for the enzyme was found to be 59 A. Molecular weight of the enzyme was then calculated to be 386,000 with the relationship M = 6πηN/1 - ρ, where η is the viscosity, N is Avogadro's number, and ρ is the density of the solvent.

Polyacylamide Gel Electrophoresis and Subunit Composition

The electrophoretic patterns of the native undissociated enzyme and of the monomeric form obtained after treatment with urea or SDS are shown in Fig. 2. The native enzyme displayed a major fast moving band, and in addition a minor slow moving band which represented 5 to 10% of the total protein. Dissociation of the same preparations into monomeric form by treatment with urea or with SDS, produced only a single band (Fig. 2). Since the electrophoretic mobilities of proteins in gels depend in the presence of urea on charge, and in the presence of SDS on molecular weight, the finding of only one band under both these conditions strongly suggests that the subunits of retinal glutamine synthetase are identical. These results also indicate that the minor band observed in the electrophoretic pattern of the native enzyme is unlikely to be due to an impurity in the preparations and may represent an aggregated form of the enzyme. Further information on this point was sought in the following experiments.

Preparations of the purified enzyme were chromatographed on Sephadex G-200 column or sedimented in 5 to 20% sucrose gradients. When the enzyme activity was assayed in the column eluent or in the gradient fractions, only a single peak of activity was observed; however, when the fractions from the sucrose gradient were assayed for protein, a small protein peak sedimenting faster than the bulk of the native enzyme was detected (Fig. 3). We assume that this small peak corresponds to the minor band observed on the electrophoretic pattern of the native enzyme. The exact nature of this component is not clear; its slower mobility relative to the major band and its apparently identical subunit composition suggest that it represents an aggregated form of the native enzyme; this conclusion is supported by the presence of small aggregates of the native enzyme in electron micrographs (see below).

The molecular weight of the subunit was determined to be 42,000 ± 2,000 from SDS-polyacrylamide gel electrophoresis,
with bovine serum albumin, ovalbumin, and chymotrypsinogen as standards (Fig. 4). Although a comparison of this value with the molecular weight of the native enzyme (392,000) would indicate the presence of nine subunits, an octameric structure is suggested by the electron micrographs of purified enzyme preparations (see below) which show a box-like structure similar to that of the octameric sheep brain glutamine synthetase.

**Electron Microscopy**

The morphology of the retinal glutamine synthetase molecules was examined following negative staining, as described under “Materials and Methods.” Fig. 5 demonstrates the excellent overall homogeneity of the purified preparation of the enzyme. The only particles noticeable are those assumed to be the enzyme, and no other macromolecular structures were observed on the grid. The individual molecules most frequently appear as compact rectangular structures with an “H”-like shape. In certain molecules, substructure can be detected within the lateral arm of each “H”; however, the present data are insufficient to propose a definitive three-dimensional, low resolution model of the molecule. Occasionally, the individual enzyme molecules were seen associated into aggregates in the form of chains (Fig. 5, inset). Both side-to-side and end-to-end aggregates were observed.

Particle dimensions were estimated from plates containing as an internal standard either tobacco mosaic virus or tropomyosin (Fig. 6); similar estimates were derived in both cases. The cylindrical tubules of tobacco mosaic virus are approximately twice the diameter of the enzyme particles. We have measured the length and width of 100 enzyme molecules in the “H” projection. The minor axis measured 90 ± 7 Å (mean ± S.D.), the major axis 127 ± 10 Å. These electron microscopic results are compatible with a three-dimensional rectangular structure, 90 × 90 × 125 Å. The size of the subunits or monomers of glutamine synthetase cannot be measured in our micrographs with adequate precision, but if the molecule contains eight subunits, one at each corner of a rectangular structure, then the monomers are probably no larger than 45 × 45 × 65 Å.

**Amino Acid Composition and Related Studies**

The amino acid composition of the chicken retina glutamine synthetase (Table II) showed close similarities with that of the enzyme from sheep brain or bacteria; however, the differences in the content of glutamic acid, histidine, and leucine show that these enzymes are not identical. The partial specific volume of the retinal enzyme was calculated from the amino acid composition (23) to be 0.725 at 20°. This value was used in the determination molecular weight of the native enzyme. Attempts to determine the NH₂-terminal residue of retinal enzyme in a Beckman amino acid sequencer with the use of the phenyl iso-
Fig. 5 (left). This figure and Fig. 6 are electron micrographs of enzyme preparations negatively stained with uranyl acetate. In both cases the magnification refers to that of the final print. This figure shows the typical configuration of the enzyme molecules with uranyl acetate negative staining. An arrow points to the “H”-shaped configuration, which is most frequently observed. The inset illustrates an area in which the molecules have aggregated side-by-side. Calibration bar = 2000 Å; magnification × 140,000.

Fig. 6 (right). A typical field of a preparation of glutamine synthetase with tobacco mosaic virus added for internal calibration and measurement of the size of the enzyme particles. Calibration bar = 1000 Å; magnification × 220,000.

Other Properties

The effect of increasing concentrations of urea on the activity of retinal glutamine synthetase is shown in Fig. 7. The enzyme is almost completely and irreversibly inactivated by concentrations of urea higher than 3 M, which, as in the case of sheep brain enzyme (4) presumably dissociates the enzymes into monomeric, catalytically inactive subunits.

The absorption spectrum of retinal glutamine synthetase shows a peak at 280 nm and a trough at 250 nm with a $A_{280}:A_{250}$ ratio of 1.6 suggesting that, unlike the E. coli enzyme (24), the retina enzyme does not contain covalently bonded AMP.

Discussion

Information concerning the characteristics of retinal glutamine synthetase is of interest for several reasons. First, by adding to what is already known about glutamine synthetase from mammalian brain and liver, and bacteria (8) it extends our knowledge about this group of proteins; second, as explained in the introduction, the importance of the hormonal induction of glutamine synthetase in the embryonic retina as a model system for studying control mechanisms in differentiation makes further knowledge of the characteristics of the retinal enzyme essential (see also Reference 25). As a step in this direction, this paper describes the purification of glutamine synthetase from the chicken neural retina, its molecular weight, subunit composition, and other physicochemical properties.

The procedure employed here for the isolation of the enzyme is a modification of that used for its purification from sheep brain (3). The acid precipitation step at pH 4.3 has been replaced, in the case of the retinal enzyme, by ammonium sulfate fractionation, since this resulted in a relatively greater increase of specific activity.

The specific enzyme activity in the 100,000 x g supernatant from the sonicate of the retina is considerably higher than that in the brain or liver; therefore, a 10-fold purification sufficed for obtaining the enzyme in a homogeneous form. The yield of the purified enzyme was approximately 1 mg from 400 mg of lyophilized retina tissue; to obtain this amount of tissue, approximately 100 adult chicken eyes had to be dissected; this imposes a certain limitation on routine large scale preparation of highly purified retinal glutamine synthetase.

Previous studies have shown that bacterial glutamine synthetase is dodecameric with a subunit molecular weight of 50,000 (6, 7); the enzyme from sheep brain, rat liver, and pea seeds is apparently octameric with subunit molecular weights estimated to be 49,000, 44,000, and 45,000, respectively (8). In the present study, the molecular weight of the subunit of the retinal enzyme was determined by SDS-polyacrylamide gel electrophoresis to be 42,000 ± 2,000; this value is in the general range for those reported for glutamine synthetase from other eukaryotic cells; furthermore, it is consistent with previous observations that in the retina, the enzyme is synthesized by polysomes estimated to comprise 12 to 14 ribosomes (12). Following dissociation of the purified enzyme into monomeric form by urea or by SDS, analysis in polyacrylamide gels yielded a single protein component; this indicates strongly that the subunits are identical, and is in agreement with the findings of subunit
Table II
Amino acid composition of glutamine synthetase from different sources

| Amino acid   | Chicken retina | Sheep brain | Bacillus subtilis |
|--------------|----------------|-------------|------------------|
| Aspartic acid| 35             | 44          | 44               |
| Threonine    | 17             | 22          | 20               |
| Serine       | 24             | 27          | 20               |
| Glutamic acid| 31             | 48          | 51               |
| Proline      | 20             | 27          | 20               |
| Glycine      | 35             | 45          | 26               |
| Alanine      | 24             | 32          | 40               |
| Half-cysteine| ND*            | 12          | 4                |
| Valine       | 19             | 20          | 26               |
| Methionine   | ND*            | 12          | 12               |
| Isoleucine   | 15             | 23          | 26               |
| Leucine      | 20             | 24          | 37               |
| Tyrosine     | 14             | 17          | 16               |
| Phenylalanine| 20             | 23          | 29               |
| Lysine       | 18             | 27          | 29               |
| Histidine    | 19             | 13          | 12               |
| Arginine     | 24             | 27          | 23               |
| Tryptophan   | ND*            | 7           | ND               |
| Literature reference | Present | Ronzio et al. (3) | Deuel et al. (7) |

* ND, not determined.

Fig. 7. Effect of urea on the activity of retinal glutamine synthetase (GS). The enzyme was incubated at 25°C for 3 hours in solutions containing 0.005 M phosphate buffer, pH 7.8, 5 mM β-mercaptoethanol, and urea, as indicated in the figure. ○—○, in these tests urea concentration in the enzyme assay mixture was 15% of that in the incubation mixture; △—△, in these tests urea concentration in the assay mixture was 1/10 of that in the incubation mixture.

Identity in sheep brain enzyme (3). The evidence for subunit homogeneity in retinal glutamine synthetase is important in considerations of genetic controls of this enzyme synthesis and regulation; however, it should be pointed out that the identity of the subunits in the retinal glutamine synthetase could not be as yet confirmed by NH2-terminal analysis, since the NH2-terminal end appears to be blocked. In contrast, the sheep brain enzyme has been reported to contain a free NH2-terminal arginine (3).

The amino acid compositions of glutamine synthetase from chicken retina, sheep brain, and bacteria (Table II) display considerable similarities, which raises the possibility of their evolutionary derivation from a common gene. Previous studies have demonstrated immunological similarities between glutamine synthetase from sheep brain and from neural retina of chick embryos (14). The high aspartate content of the retinal enzyme explains the preferential incorporation of this amino acid into glutamine synthetase in retina cells which are actively synthesizing the enzyme due to hormonal induction (13).

The discrepant results of the various determinations of molecular weight of retinal glutamine synthetase should be examined. The value obtained by sedimentation equilibrium was 322,000, and by sedimentation velocity and gel permeation was 386,000. However, accepting the subunit molecular weight to be 42,000 and the octameric form of the enzyme, the calculated molecular weight is 336,000. These disparate results may be due to inaccuracies inherent in all three methods of measurements used. Thus, measurements of subunit molecular weight by gel electrophoresis have an error of about 5%, a value that could account for some of the difference between the expected and the observed molecular weights of the octamer. In the case of sedimentation equilibrium measurements, the presence in solution of aggregates or stacks of the enzyme octamers, such as seen in the electron micrographs (Fig. 5, inset) could have artificially raised the value. It can be calculated, for instance, that the presence of 16% of an octamer doublet (calculated mol wt 672,000) would suffice to raise the weight average molecular weight from the expected 336,000 to the observed value of 392,000. There is, at present, no way to determine whether such aggregates persist even at very high dilutions. Aggregation and stacking of the native enzyme was also found in glutamine synthetase isolated from bacteria (25). Thus, in the face of the electron microscopic and other physiochemical data available to us at present, it seems reasonable to conclude that the molecular weight of the chicken retina glutamine synthetase is of the order of 340,000, and that this enzyme consists of 8 apparently identical subunits.

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Vol. 247 (1972) 7743-7749

In SARKAR, PRANAB K., DONALD A. FISCHMAN, EUGENE GOLDWASSER, and ARON A. MOSCONA. Isolation and Characterization of Glutamine Synthetase from Chicken Neural Retina.

Page 7745, Paragraph 1 under "Molecular Weight of Native Enzyme," Line 4 should read:

(0.375, 0.55, and 0.75 mg per ml) in 0.1 M phosphate buffer,

Vol. 248 (1973) 528-533

In Yu, C. A., L. Yu, and Tsoo E. King. Kinetics of Electron Transfer between Cardiac Cytochrome c1 and c.

Page 531, left-hand column, Lines 9, 11, and 22, $\times 10^5$ M$^{-1}$ s$^{-1}$ should read:

$10^6$ M$^{-1}$ s$^{-1}$. In all other places, including the Summary, the data are correctly printed.

Vol. 248 (1973) 472-477

In OWENS, IDA S., BARBARA K. VONDERHAAR, and YALE J. Topper. Concerning the Necessary Coupling of Development to Proliferation of Mouse Mammary Epithelial Cells.

Page 475, Table VI, Column 4, the heading should read:

$\Delta$ Casein per 10$^4$ cells.

Due to a mishap in printing the $\Delta$ was omitted.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.