Regional effects of sodium aspartate and sodium glutamate on protein synthesis in the retina

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Isolated retinas from Xenopus laevis tadpoles or juvenile frogs were incubated in Ringer's bicarbonate-glucose medium in which sodium chloride was replaced with equimolar amounts of either sodium aspartate (NaAsp) or sodium glutamate (NaGlu). At all concentrations tested (0.1 to 50 mM), both acidic amino acids caused a dramatic decrease in the incorporation of $^3$H-leucine into proteins in the inner retina. Higher concentrations of these acids (50 mM) caused an increase in the incorporation of the label by photoreceptors. Polyacrylamide gel electrophoresis of whole retinal proteins showed a selective increase in incorporation of the label into rhodopsin, a protein unique to rod photoreceptor cells. Quantitative autoradiographic and biochemical studies showed that NaAsp had no effect on the uptake of $^3$H-leucine by the retinal cells, suggesting that the effect is directly on protein synthesis. When retinas were removed from NaAsp treatment, protein synthetic capacity recovered, and the degree of recovery was related to the concentration of NaAsp in the preincubation medium. These studies demonstrate that acidic amino acids in concentrations utilized in electrophysiologic studies to isolate the photoreceptor generated a-wave of the electroretinogram also have a profound effect on retinal protein synthesis. Whether these two actions are related remains to be determined. (Invest Ophthalmol Vis Sci 21:554-562, 1981.)

Key words: retina, sodium aspartate, sodium glutamate, protein synthesis

In 1955 Furukawa and Hanawa observed that sodium aspartate (NaAsp) and sodium glutamate (NaGlu) abolished the b-wave of the electroretinogram. Since that time, NaAsp has been used extensively in electrophysiologic studies as a means of isolating the photoreceptor-generated a-wave, although the mechanism of this action remains unexplained. During a series of in vitro experiments with retinas from the clawed toad, Xenopus laevis, we observed dramatic alterations in the pattern of protein synthesis in various retinal layers in response to treatment with NaAsp or NaGlu. Using biochemical and autoradiographic techniques to monitor protein synthesis, we noted that acidic amino acids greatly reduced the incorporation of labeled leucine into protein in cells of the inner retina, whereas in the photoreceptors, protein synthesis was enhanced above control levels at the highest concentration tested (50

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These studies were supported in part by grants EY-00871, EY-07001, EY-02420, and EY-02365 from the National Eye Institute, National Institutes of Health, Bethesda, Md., and by the Retina Research Foundation, Houston, Texas; Research to Prevent Blindness, Inc., New York, N.Y.; the Brown Foundation, Houston, Texas; and a Center Grant from the National Retinitis Pigmentosa Foundation, Baltimore, Md.

Submitted for publication Dec. 1, 1980.

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554 0146-0404/81/100554+09$00.90/0 © 1981 Assoc. for Res. in Vis. and Ophthal., Inc.
mM). A preliminary report of these studies has been presented.

Methods
Juvenile frogs or tadpoles were maintained for at least 1 week in constant-temperature incubators (21°C) on a controlled lighting cycle (lights on at 9:00 A.M. and off at 9:00 P.M.). On the day of an experiment, animals were kept in the dark and retinas were dissected from the pigment epithelium under dim red light. Dissections were begun at 9:00 A.M. and were usually completed within 30 min. Pooled retinas were incubated in the dark in a modified Ringer's bicarbonate-glucose medium (RBG) containing 120 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 1.25 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, 16 U/ml penicillin G, and 24 μg/ml streptomycin, which had been gassed with 95%O₂:5%CO₂ in the cold for 15 to 20 min just prior to use. In some incubations NaCl was replaced with equimolar amounts of NaAsp, NaGlu, or sodium isoleucine (Nalle). Puromycin was added to some incubations to inhibit protein synthesis (50 μg/ml). After a 30 min preincubation in the appropriate RBG, L-14C- or L-3H-leucine (1 to 2 μCi/ml; final concentration in all incubations, 3.2 μM) was added and the incubations continued in the light for the appropriate intervals.

For biochemical studies of protein synthesis, retinas were treated as described above and were then incubated in the light for 2 hr at 21°C in 10 ml of RBG with constant gassing (1 cu ft/hr/20 cm² of surface area). The reaction was terminated by rapidly rinsing the retinas three times in the appropriate cold RBG and placing individual retinas in ice-cold 10% trichloroacetic acid (TCA) for 30 min. After one wash with deionized water at room temperature, retinas were either solubilized in 1N NaOH for protein determination or prepared for polyacrylamide gel disc electrophoresis. Aliquots of the Lowry protein solutions were counted for the determination of specific radioactivity of protein. Each polyacrylamide gel was stained with Coomassie blue and scanned at 550 nm. Ten regions were identified and taken for the determination of radioactivity. Band 6 in Fig. 7 corresponds to opsin, identified in a pure preparation of rod outer segments from X. laevis.

For autoradiographic analysis, incubated retinas were rinsed three times with the appropriate cold RBG and were then fixed in 0.1M cacodylate buffer (pH 7.4) and 0.025% CaCl₂ containing either 3% formaldehyde plus 3% sucrose or 2% glutaraldehyde. The formaldehyde used as a fixative after incubations with radioactive amino acids retains only that radioactivity which has been incorporated into protein, whereas glutaraldehyde retains both incorporated and unincorporated radioactivity within the retinal cells. Exposure time for the autoradiograms was 5
Fig. 2. Regional distribution of the grain counts from the autoradiographs presented in Fig. 1. Absolute grain counts per 105 μm² are plotted on the ordinate vs. region of the retina from which the counts were obtained. The underlying micrograph aids in the specific identity of each area. Note that for retinas incubated in 50 mM NaAsp (●) and 50 mM NaGlu (△) there is a decrease in grain counts across the entirety of the inner retina, with an increase over the photoreceptors compared to controls (○). Incubation in 50 mM Nalle (□) resulted in a uniform reduction in silver grains across the entire retina.

Days, except for some of the retinas incubated with the Nalle, which required 77 to 84 days of exposure. Quantitative grain counts were made directly from the autoradiographs with a Zeiss MOP-3 interfaced to a standard microscope through a drawing tube attachment.

For studies of uptake of labeled leucine into the retina, incubations were for 5, 10, and 15 min at room temperature in pregassed media. At the indicated times individual retinas were removed from the incubation media, washed three times with cold RBG, placed immediately in ice-cold 10% TCA for 30 min, and washed once with deionized water. An aliquot of the combined TCA and water washings was taken for determination of TCA-soluble radioactivity, and the TCA-insoluble protein was assayed and counted. Both uptake of radioactive leucine into the cell (TCA-soluble) and incorporation into protein (TCA-insoluble) are expressed as disintegrations per minute per microgram of TCA-insoluble retinal protein.

Results

Effects of amino acids on the regional incorporation of 3H-leucine into retinal protein. Incubation of isolated X. laevis retinas with 3H-leucine in standard RBG media resulted in the incorporation of significant amounts of radioactivity into retinal proteins. Replacement of 50 mM NaCl with 50 mM NaAsp resulted in at most a 25% decrease in the amount of label incorporated over a 2 hr period, expressed as disintegrations per minute per microgram of retinal protein. However, a profound effect of NaAsp and NaGlu on the regional distribution of radioactivity in retinal proteins was observed. In the dark-field autoradiograms presented in Fig. 1 it can be clearly seen that silver grains are present across the entire expanse of retinas incubated in the control media. All layers are labeled to some extent, although there appears to be a greater concentration of silver grains in the region of the photoreceptors. Retinas...
incubated with 50 mM NaAsp showed a paucity of silver grains over the inner retina and an apparent increase in silver grains over the photoreceptors. A virtually identical distribution was observed for 50 mM NaGlu. The Nalle-treated retinas, when exposed for autoradiography for the same length of time as controls, contained few silver grains.

Quantitation of the regional distribution of the silver grains in these autoradiographs is presented in Fig. 2. Grain counts per 105 μm² are plotted on the ordinate vs. the region of the retina from which the counts were obtained. The underlying micrograph helps to identify the different retinal areas. At least three different regions from three sections of three individual retinas were examined for each treatment indicated, and all data points represent the mean grain counts per retinal area. For this analysis all retinas were incubated under the same conditions and autoradiograms were developed after the same length of exposure time.

In the control retinas the ganglion cell layer and the outer plexiform layer contained the highest number of silver grains in the inner retina, and these counts were consistently higher than those in retinas incubated with NaAsp, NaGlu, or Nalle. In the outer retina the inner segments of the photoreceptors showed the highest density of silver grains. The lowest level of incorporation of label appeared to be in the outer segment, which is to be expected, since these incubations were for only 2 hr and these organelles do not synthesize protein. Although incubation in 50 mM NaAsp or 50 mM NaGlu resulted in dramatic reductions in the number of silver grains across the inner retina compared with controls, there was an increase in silver grains over the inner segments of the photoreceptor cells incubated with these acidic amino acids.

In retinas incubated in NaIle, only small amounts of radioactivity were detectable when autoradiographs were exposed for the same length of time as controls. With longer exposure periods (77 to 84 days) regional distribution of radioactivity was identical to that observed in controls.

The ratios of grain counts of NaAsp, NaGlu, and NaIle to control retinas are presented in Fig. 3. This treatment emphasizes the effect of the acidic amino acids in reducing incorporation of the label into ganglion cell and inner plexiform layer proteins while at the same time increasing the incorporation of label into proteins of the photoreceptor cells.

**Effect of acidic amino acids on the uptake of 3H-leucine into the retina and incorporation into protein.** We tested the possibility that the regional effects of NaAsp on the incorporation of labeled leucine into retinal proteins were caused by a difference in uptake of 3H-leucine into the cells. In Fig. 4 is plotted the TCA-soluble radioactivity from incubations in control media or in media in which 50 mM NaCl was replaced with 50 mM NaAsp. There was no apparent difference in the uptake (TCA-soluble) of 3H-leucine in the control or in the media containing NaAsp, indicating that NaAsp does not affect the uptake of the label into the total retina. There was, however, a profound effect of NaIle on 3H-leucine uptake, as would be predicted, since both of these amino acids use the same uptake channel.

As shown in Fig. 5, once the 3H-leucine entered the retinal cells, the tracer was incorporated into newly synthesized protein.
Fig. 5. Incorporation of $^3$H-leucine into TCA-precipitable material from the same retinas used to measure uptake (Fig. 4). No differences were noted between control (○) and 50 mM NaAsp (●) retinas. Only small amounts of radioactivity were incorporated in the retinas incubated with 50 mM Nalle (□).

There was no apparent difference between the rates of incorporation of the label into protein in the retinas incubated for these short times in NaAsp and those incubated in control media.

Although the biochemical evidence indicated no effect of NaAsp on the bulk uptake of $^3$H-leucine into the retina, the possibility existed that there was a regional effect similar to the one observed for protein synthesis. Therefore, to determine the regional distribution of $^3$H-leucine uptake we analyzed by autoradiography the distribution of radioactivity in retinas in which protein synthesis was inhibited with puromycin. This maneuver allowed us to observe uptake only.*

After 15 min of incubation, retinas were thoroughly rinsed to remove all extracellular radioactivity and were fixed in either glutaraldehyde-formaldehyde (to cross-link and retain all intracellular $^3$H-leucine during subsequent tissue processing) or in formaldehyde (which retains only that $^3$H-leucine previously incorporated into protein). Quantitative autoradiography across the retina revealed the distribution shown in Fig. 6. There were no regional differences between the NaAsp-treated and control retinas with regard to incorporation of radioactivity into protein in the presence of puromycin (formaldehyde fixation). Nor were there any significant regional differences in the distribution of radioactivity in the glutaraldehyde-fixed material. The slight decrease observed in the NaAsp-treated retinas across the inner retina was not significant and could not account for the dramatic inhibition of label incorporation by NaAsp treatment evident in Figs. 2 and 3. Furthermore, the enhancement by NaAsp of the incorporation of tracer into the photoreceptor proteins is not accompanied by an increased uptake of $^3$H-leucine by these cells, since grain counts over these strata are identical in control and NaAsp-treated retinas. Therefore we conclude that the effect of NaAsp on the regional synthesis of proteins in the retina is not the result of a differential uptake of $^3$H-leucine by retinal neurons.

Incorporation of radioactive leucine into cell-specific proteins. The implication of the biochemical and autoradiographic studies is that NaAsp causes a decrease in protein synthesis in the inner retina and a stimulation of protein synthesis in the photoreceptors. To test this hypothesis we determined whether NaAsp treatment caused an increase in the incorporation of $^3$H-leucine into a photoreceptor-specific protein. The visual pigment, rhodopsin, is useful for this type of analysis, since it is synthesized only by rod photoreceptor cells. Retinas were incubated with either $^{14}$C- or $^3$H-leucine in control media or in media containing 50 mM NaAsp. After 2 hr, single retinas from the following incubation flasks were mixed: (1) $^{14}$C-control and $^3$H-control, (2) $^{14}$C-control and $^3$H-NaAsp, (3) $^{14}$C-NaAsp and $^3$H-control, and (4) $^{14}$C-NaAsp and $^3$H-NaAsp. Each pair of retinas was denatured with TCA as described previously, and the proteins were solubilized and subjected to polyacrylamide gel disc elec-
Aspartate effects on retinal protein synthesis

Fig. 6. Regional distribution of the grain counts from autoradiograms of retinas incubated for 15 min with $^3$H-leucine in control media (circles) or media containing 50 mM NaAsp (triangles) in the presence of puromycin (50 $\mu$g/ml). Retinas were fixed with glutaraldehyde (closed symbols) to retain all intracellular radioactivity, or formaldehyde (open symbols) to retain only radioactivity incorporated into protein. There were no significant regional effects of NaAsp on the uptake of leucine into the retinal cell layers. GL, Ganglion layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PL, photoreceptor layer; IS, inner segment; OS, outer segment.

Fig. 7. Polyacrylamide gel disc electrophoresis of retinas incubated with either $^3$H-leucine or $^{14}$C-leucine in control media or media containing 50 mM NaAsp, and paired as described in the text. The values are the average of combinations 3 and 1, and 4 and 2, determined as described in the text. Band 1 contains the highest molecular weight proteins. There was a selective incorporation of radioactivity into the band containing opsin (band 6), which was consistent with the concept of an increased protein synthesis in the rods, suggested from the autoradiography data (Figs. 2 and 3).

trrophoresis. Ten distinct regions were distinguished in these gels (Fig. 7). The gels were divided according to these regions, solubilized in $H_2O_2$, and counted for radioactivity, and the ratio of $^{14}$C/$^3$H was determined. Combinations 1 and 4 are controls whose normalized ratios should be equal to one, whereas any effect of NaAsp should be reflected in the ratios of combinations 2 and 3. By dividing the ratios obtained in combination 3 by those in combination 1, and those in combination 4 by those in combination 2, we obtained the ratios of $^{14}$C-leucine incorporated in the presence of 50 mM NaAsp to control retinas incubated in the absence of NaAsp. This algebraic maneuver corrected for any deviations from unity in the controls. Ratios greater than one indicate an increased incorporation of radioactivity in the presence of 50 mM NaAsp, whereas ratios less than one indicate a decrease in the incorporation into protein.

As can be seen in Fig. 7, NaAsp depressed the incorporation of label into higher molecular weight proteins but enhanced the incorporation into opsin (band 6). These data are consistent with our autoradiographic observations that the incorporation of radioactivity into photoreceptor cells is enhanced by NaAsp. We cannot determine the cell of origin of any of the other nine protein bands, and indeed all ten bands probably contain proteins from every retinal cell. However, the relative increase in the labeling of opsin, the major protein in the photoreceptor cell, is clear biochemical evidence that protein synthesis has been differentially affected in the retinas treated with NaAsp. Whether this increase is specific for opsin alone remains to be determined.

Effect of concentration of NaAsp and NaGlu on the regional synthesis of protein in the retina. The effect of 0.5, 5.0, and 50 mM concentrations of NaAsp and NaGlu on the regional incorporation of radioactivity into retinal proteins is demonstrated in Figs. 8 and 9, respectively. At all concentrations there was a reduction in the incorporation of
Fig. 8. Effects of the concentration of NaAsp on the regional distribution of radioactivity in retinal proteins after incubation with \(^{3}\)H-leucine. Concentrations ranging from 0.5 to 50 mM resulted in the depression of protein synthesis in the inner retina, whereas only the higher concentration appeared to stimulate incorporation into proteins of the photoreceptor layer.

\(^{3}\)H-leucine into protein by cells of the inner retina. However, only at the higher concentrations was the enhancement of incorporation of radioactivity into photoreceptor cell protein observed. The effects of both amino acids appear to be similar in most regions of the retina.

**Recovery or protein synthetic activity after removal of NaAsp.** Retinas preincubated for 30 min in increasing concentrations of NaAsp followed by 2 hr in control media containing labeled leucine demonstrated by quantitative autoradiography that inhibition of protein synthesis by NaAsp is reversible (Fig. 10). Recovery of some synthetic capability in the inner retina was observed at all NaAsp concentrations but was greatest at the lowest concentration. At 0.1 mM NaAsp the recovery reached control values.

**Discussion**

The effects of acidic amino acids on the developing retina are well known.\(^7\)–\(^9\) When glutamate is added to the diet of weanling rodents, specific lesions occur in the inner retina. Although the mechanism of action of glutamate is not known, the observations in this paper of a marked reduction of protein synthesis in inner retinal cells when mature retinas were incubated in the presence of acidic amino acids suggest the possibility that in the developing system, protein synthesis may be abolished by glutamate treatment, which subsequently leads to the degeneration of inner retinal cells. In the present study, no morphologic differences were observed between retinas incubated for up to 2 hr in control media or media in which NaCl had been replaced with NaAsp or NaGlu.

NaAsp and, to a lesser extent, NaGlu have also received extensive use as means of isolating the a-wave of the electroretinogram (ERG). A large range of concentrations has been used to accomplish this perturbation.\(^11\)–\(^17\) In the present study we have shown that incubating retinas of *X. laevis* in organ culture with NaAsp or NaGlu at concentrations ranging from 0.5 to 50 mM alters the normal pattern of protein synthesis. At the highest concentration these acidic amino acids increase incorporation of labeled leucine into protein in the photoreceptors while simultaneously decreasing the incorporation of the tracer by cells in the inner retina. This differential effect is not so apparent from biochemical studies of whole retinas where disintegrations per minute per microgram of retinal protein is measured (Fig. 5), probably be-
cause the decrease in incorporation in the inner retina is offset by an increase in incorporation into photoreceptors. However, the regional differences between control and treated retinas is readily apparent after quantitative autoradiography (Figs. 1 to 3). The stimulation of incorporation of tracer into photoreceptors is lost at the lower concentrations of NaAsp and NaGlu. Since the suppression of incorporation into proteins in the inner retina is observed at all concentrations, it is likely that these two effects are separate.

The biochemical studies measuring the effects of NaAsp on uptake of the labeled precursor showed no differences from controls. The autoradiographic studies of retinas incubated in the presence of puromycin clearly demonstrated that all cells took up the label, and no significant differences were observed in any retinal cell layer between the control and NaAsp-incubated retinas. Therefore the effect of NaAsp is not on the uptake of labeled leucine but rather on the incorporation of this tracer into protein.

Biochemical studies after the incorporation of labeled leucine into a cell-specific protein, the visual pigment apoprotein opsin, also clearly demonstrated a stimulation of protein synthesis in photoreceptor cells by NaAsp as compared with controls. Whether this incorporation is specific only for opsin could not be determined, since it is possible that other photoreceptor-specific proteins may also have been stimulated but could not be identified on the gel. Presumably most of the bands on the gel represent proteins of similar molecular weights from various retinal cell types. The reason we were able to see the difference in the opsin band is that opsin is the major protein of the X. laevis retina and comprises most, if not all, of the protein in band 6.

Our biochemical data can be compared with those of Reif-Lehrer, et al. who studied the effects of NaGlu on the morphology and protein synthesis in the 12-day chick embryo retina. They found that retinas cultured for 5 or 24 hr in 2.4 mM NaGlu containing 14C-phenylalanine incorporated 15% to 35% less radioactivity into retinal proteins than did control retinas. Unlike our mature X. laevis retinas the chick embryo retinas had undergone rather severe degenerative changes in the inner retina so that it was not possible to determine whether the decrease in protein synthesis was actually due to an effect of NaGlu or was secondary to cell damage.

We do not know how NaAsp causes the decrease in protein synthesis in the cells of the inner retina. One possibility may be related to the changes in energy metabolism in the various parts of the retina because of depolarization of second order neurons. Dowling and Ripps and Cervetto and McNichol have shown that the action of NaAsp on horizontal cells is to depolarize the cells. That these cells become unresponsive to light indicates that the ionic gradients across the retina which are necessary to generate an electrical response have been altered. These ionic gradients are maintained by specific pumps that require adenosine triphosphate (ATP) as a source of metabolic energy. If

Fig. 10. Recovery of protein synthetic activity in NaAsp-treated retinas after 30 min preincubation in various concentrations of NaAsp. Each retina was incubated for an additional 2 hr in RBG (control media) or the preincubation media containing 3H-leucine. Quantitative grain counts determined from autoradiograms are plotted vs. retinal layers. Open symbols represent control retinas (○) or retinas incubated continuously in the presence of 0.1 mM (△) or 10 mM (□) NaAsp. Closed symbols represent retinas preincubated in 0.1 mM (●) or 10 mM (▲) NaAsp for 30 min, followed by a 2 hr incubation in control media.
these gradients are destroyed, the requirement for energy to reestablish the gradients would be tremendous, and perhaps the entire energy produced by these depolarized cells would now be directed towards this function. Thus the ATP and guanosine triphosphate normally available for protein synthesis would not be available in those cells that were depolarized by NaAsp.

Retinas bathed in 50 mM NaAsp recover electrical activity in about 10 to 20 min. Protein synthesis in the inner retinas of X. laevis also recovers after removal of NaAsp in a dose-dependent manner (Fig. 10). Although the time course of recovery was not determined, it must be somewhat less than 2 hr, the length of time of our incubations with labeled leucine. Since protein products of gene transcription do not usually appear for at least 2 hr after induction, the effect of NaAsp is most likely directly on protein synthesis rather than at the level of transcription.

The effects of NaAsp and NaGlu on the retina can be used in biochemical studies according to the same rationale applied in electrophysiologic experiments. Since high levels of these acidic amino acids abolish protein synthesis in the inner retina while at the same time stimulating synthesis in the photoreceptor cells, this preparation may prove to be a useful method for isolating photoreceptor synthesis from biosynthetic events in other retinal neurons in intact retinas.

Acidic amino acids have now been shown to have three effects on the retina. One is to abolish the b-wave of the ERG; another is to damage the inner layers of immature retinas; the third is to produce regional alterations in protein synthesis. Whether these effects are related cannot be determined from our present data. But clearly the abolition of inner retina protein synthesis in the same cells in which these amino acids are known to eliminate electrical responses raises the distinct possibility that these events may be more than coincidental.

The assistance of M. Bayborn, M. B. Maude, S. Robbins, and J. Rosenthal is greatly appreciated.

REFERENCES