Biol. 102, 613-631.
Atkinson, P. H., and Summers, D. F. (1971), *J. Biol. Chem. 246,* 5162-5175.
Bekesi, J. G., and Winzler, R. G. (1967), *J. Biol. Chem. 242,* 3873-3879.
Bennet, G., and Leblond, C. P. (1970), *J. Cell Biol. 46,* 409-416.
Bennet, G., Leblond, C. P., and Haddad, A. (1974), *J. Cell Biol. 60,* 258-284.
Cleaver, J. E. (1967), Thymidine Metabolism and Cell Kinetics, Amsterdam, North-Holland Publishing Co.
Cook, J. S., Will, P. C., Proctor, W. R., and Brake, E. T. (1976), in Biogenesis and Turnover of Membrane Molecules, Cook, J. S., Ed., New York, N.Y., Raven Press.
Devreotes, P. N., and Fambrough, D. M. (1975), *J. Cell Biol. 65,* 335-358.
Eagle, H. (1959), *Science 130,* 432-437.
Hartzell, H. C., and Fambrough, D. M. (1973), *Dev. Biol. 30,* 153-165.
Huang, C.-C., Tsai, C.-M., and Canellakis, E. S. (1973), *Biochim. Biophys. Acta 332,* 59-68.
Hubbard, A. L., and Cohn, Z. A. (1975), *J. Cell Biol. 64,* 461-479.
Kaufman, R. L., and Ginsburg, V. (1968), *Exp. Cell Res. 50,* 127-132.
Kornfeld, S., Kornfeld, R., and Ginsburg, V. (1965), *Arch. Biochem. Biophys. 110,* 1-7.
Kraemer, P. M. (1971), *Biomembranes 1,* 67-190.
Levine, E. M. (1972), *Exp. Cell Res. 74,* 99-109.
Levine, E. M., Thomas, L., McGregor, D., Hayflick, L., and Eagle, H. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 583-589.
Melchers, F., and Anderson, J. (1973), *Transplant. Rev. 14,* 76-130.
Schimke, R. T. (1975), *Methods Membr. Biol. 3,* 201-236.
Shen, L., and Ginsburg, V. (1967), *Arch. Biochem. Biophys. 122,* 474-480.
Trujillo, J. L., and Gan, J. C. (1971), *Biochim. Biophys. Acta 230,* 610-619.
Tweto, J., and Doyle, D. (1976), *J. Biol. Chem. 251,* 872-882.
Warren, L., and Glick, M. C. (1969), *J. Cell Biol. 37,* 729-746.
Yurchenco, P. D. (1975), Ph.D. Thesis, Sue Golding Graduate Division of the Albert Einstein College of Medicine, New York, N.Y.
Yurchenco, P. D., and Atkinson, P. A. (1975), *Biochemistry 14,* 3107-3114.

Differential Effects of Puromycin on the Incorporation of Precursors of Rhodopsin in Bovine Retina†

Paul J. O'Brien

ABSTRACT: Bovine retinas incubated in vitro sustain the synthesis of opsin and rhodopsin as monitored by the incorporation of labeled leucine, mannose, and glucosamine. Puromycin, an inhibitor of protein synthesis, effectively blocks the incorporation of leucine and mannose into opsin and rhodopsin of rod outer segments. However, the incorporation of glucosamine into opsin and rhodopsin is not immediately blocked. Instead, it continues for a time suggesting not only core oligosaccharide synthesis but also the secondary glycosylation of a pool of preformed opsin which is thought to be transiently accumulated in the photoreceptor Golgi complex. Galactose, not normally found in rhodopsin, is also incorporated into both opsin and rhodopsin. This incorporation appears to be competely insensitive to puromycin, suggesting that it may occur in the rod outer segments involving only preexisting glycoproteins.

The oligosaccharide component of rhodopsin consists of two types of sugar residues, mannose and *N*-acetylglucosamine (Heller, 1968; Heller and Lawrence, 1970; Plantner and Kean, 1976; Shichi et al., 1969). These sugars occur in ratios of approximately 9 mannose and 5 *N*-acetylglucosamine residues per mol of rhodopsin (Plantner and Kean, 1976) with *N*-acetylglucosamine acting as the link between the oligosaccharide and an asparagine residue of the polypeptide (Heller and Lawrence, 1970). Oligosaccharides of similar composition are found in many glycoproteins and can have a variety of structures based on the *N*-acetylglucosamine linkage to asparagine (Montgomery, 1972). These oligosaccharides are referred to as core oligosaccharides since they can serve as the base upon which more complex carbohydrate chains are assembled. The synthesis of core oligosaccharides takes place on the rough endoplasmic reticulum (Bouchilloux et al., 1970) probably as a lipid-oligosaccharide complex with the oligosaccharide ultimately being transferred to newly synthesized polypeptides (Lennarz, 1975). Puromycin inhibits protein synthesis in the rough endoplasmic reticulum and should cause a substantial inhibition of the incorporation of core oligosaccharides since the acceptor polypeptide would not be produced. Puromycin has been shown to block the incorporation of leucine into rhodopsin in both bovine (O'Brien et al., 1972) and frog retinas (Basinger and Hall, 1973). The oligosaccharide of rhodopsin has the simple composition of a core oligosaccharide and may be synthesized through a lipid-linked intermediate (Kean and Plantner, 1976). If rhodopsin has only a core oligosaccharide, then puromycin should substantially

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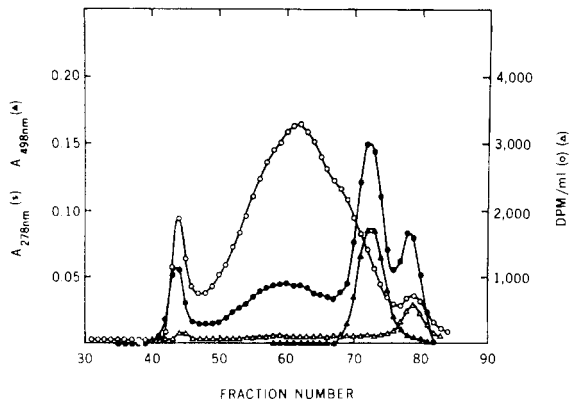

FIGURE 1: Agarose chromatography of extracts of rod outer segments from retinas incubated for 2 h with [^{3}H]leucine. The column was 1.5 × 168 cm and consisted of Bio-Gel A-1.5m, 100–200 mesh, equilibrated with 0.3% Ammonyx LO in 0.05 M Tris-HCl, pH 7.8. The flow rate was 12 ml/h and 3-ml fractions were collected. Absorbance at 278 nm (●) and 498 nm (▲) of the control outer segment extract is plotted along with disintegrations min^{-1} ml^{-1} for [^{3}H]leucine incorporated in the presence (△) and absence (○) of 10^{-4} M puromycin. The amount of rhodopsin in the outer segments from the puromycin incubation was 103% of the control.

inhibit the incorporation of both mannose and *N*-acetylglucosamine. However, evidence has been presented showing that there is incorporation of glucosamine into rhodopsin not only in the rough endoplasmic reticulum but also in the Golgi complex of photoreceptors (Bok et al., 1974) at a time substantially later than leucine incorporation (O'Brien and Muellenberg, 1974) suggesting the initiation of a terminal trisaccharide like that in many glycoproteins such as thyroglobulin (McQuillan and Trikojus, 1972). More recent experiments using isolated bovine retinas (O'Brien, 1976) have shown that galactose and fucose can also be incorporated into opsin and rhodopsin thus completing the terminal trisaccharide.

A series of experiments was carried out to determine the effect of puromycin on the incorporation of leucine, mannose, *N*-acetylglucosamine, and galactose into rhodopsin in an attempt to differentiate between core oligosaccharide and terminal trisaccharide synthesis.

Methods

Preparation and Incubation of Retinas. Bovine retinas were isolated and incubated in Krebs–Ringer bicarbonate medium with glycerol as a carbon source as previously described (O'Brien et al., 1972; O'Brien and Muellenberg, 1974). Each incubation vessel contained two retinas and one of the following radioactive precursors: 50 μCi of L-4,5-[^{3}H]leucine (41.2 Ci/mmol), 50 μCi of D-6-[^{3}H]glucosamine (7.3 Ci/mmol), 100 μCi of D-2-[^{3}H]mannose (2 Ci/mmol), 83 μCi of D-1-[^{3}H]galactose (5.95 Ci/mmol) and, where indicated, 10 μCi of D-1-[^{14}C]mannose (50.5 mCi/mmol). All isotopes were purchased from New England Nuclear, Boston, Mass., except the [^{3}H]mannose which came from Amersham/Searle, Arlington Heights, Ill. Puromycin hydrochloride was supplied by Nutritional Biochemicals, Cleveland, Ohio. Incubations were carried out at 37 °C in dim red light.

Preparation of Rod Outer Segments and Chromatography of Rhodopsin. Radiolabeled rod outer segments were isolated and purified by the method of Papermaster and Dreyer (1974) as slightly modified (O'Brien and Muellenberg, 1974). The labeled outer segments from four retinas were extracted with 3 ml of 1% Emulphogene BC 720 (General Aniline, New York, N.Y.) in 0.05 M Tris-HCl, pH 8.5, for 15 min at room temperature, and the extracts were clarified by centrifugation for 20 min at 180 000*g*. Extracts were chromatographed at 4 °C on a 1.5 × 168 cm column of Bio-Gel A-1.5m, 100–200 mesh, equilibrated with 0.03% Ammonyx LO (Onyx Chemical Co., Jersey City, N.J.) in 0.05 M Tris-HCl, pH 7.8. The flow rate was 12 ml/h and was controlled by a metering pump with 3-ml fractions being collected. Spectrophotometric and radioactivity measurements of column fractions were as previously described (O'Brien and Muellenberg, 1974; O'Brien et al., 1972). All operations were carried out in the dark or in dim red light.

In experiments demonstrating the conversion of [^{3}H]glucosamine labeled opsin to visual pigment, labeled outer segments were incubated with 9-*cis*-retinal (Eastman) as previously described (O'Brien and Muellenberg, 1975). Extraction and chromatography was as in the other experiments, except that the eluting detergent was 1% Emulphogene BC-720 (General Aniline) causing rhodopsin to be eluted at fraction 60 instead of fraction 70 on the agarose column.

Results

Inhibition of Rhodopsin Labeling by Puromycin. Groups of four bovine retinas were incubated for various times in the presence or absence of 10^{-4} M puromycin with either [^{3}H]leucine, [^{3}H]mannose, or [^{3}H]glucosamine as the labeled precursor. Rod outer segments from four retinas were purified and extracted with Emulphogene. Each extract was chromatographed on agarose and the absorbances at 278 and at 498 nm, the absorption maxima of rhodopsin, were measured as was the radioactivity in 1 ml of each 3-ml fraction. Comparable amounts of rhodopsin were chromatographed to allow direct comparison. Identical elution patterns were obtained with the control outer segments and those from retinas incubated with puromycin. The effect of puromycin on leucine incorporation at 2 h is shown in Figure 1. It can be seen that in the presence of puromycin virtually no radioactivity was incorporated either into opsin, the broad peak at fraction 60 in the control, or into rhodopsin at fraction 72. (It has been shown that 11-*cis*-retinal treatment of outer segments prior to extraction converts the broad peak at fraction 60 to one migrating with rhodopsin, thus identifying it as opsin (O'Brien and Muellenberg, 1975)). A minor component trailing rhodopsin, at fraction 79, is, however, unaffected by puromycin.

A similar pattern was produced when [^{3}H]mannose incorporation for 3 h was blocked by puromycin (Figure 2). These results suggest that mannose is added to newly synthesized opsin either as the polypeptide is being elongated or shortly thereafter and that no significant pool of mannose-free opsin ever accumulates. Such results would be consistent with a mechanism whereby the oligosaccharide is synthesized independently, bound to a lipid, and is subsequently transferred to opsin. This mechanism would predict that glucosamine, the residue that links the oligosaccharide to the polypeptide should also fail to label opsin and rhodopsin in the presence of puromycin. However, such was not the case. Figure 3 shows that puromycin caused only a partial blockage of glucosamine incorporation during a 2-h incubation. Unlike leucine and mannose, significant labeling with glucosamine still occurred in the presence of puromycin. Thus, transfer of some *N*-acetylglucosamine residues to opsin must occur after the initial addition of the core oligosaccharide.

More extensive studies were carried out to determine more precisely the nature of this secondary labeling with glucosamine. The experiments were carried out as before and the results are summarized in Table I. It can be seen that puromycin

TABLE I: Effect of Puromycin on the Specific Activity of Rhodopsin and Opsin.[a]

Precursor	Incubation Time (h)	Sp Act. (% of Control)	
		Rhodopsin	Opsin
[^{3}H]Leucine	2	904 ± 123 (4.3)	1 323 ± 21 (1.6)
	4	1 064 ± 125 (2.5)	2 267 ± 34 (1.1)
[^{3}H]Mannose	3	277 ± 65 (9.1)	1 151 ± 26 (3.2)
	4	492 ± 187 (13)	613 ± 20 (5.0)
[^{3}H]Glucosamine	2	11 467 ± 219 (71)	25 021 ± 92 (32)
	4	13 083 ± 177 (27)	27 581 ± 75 (12)

[a] Specific activity is defined as dpm per ml per A_{498nm} for rhodopsin and dpm per ml per A_{278nm} for opsin. The standard deviation is shown for each dpm determination. The control deviations were generally less than 1% except for mannose which showed 3.6 and 3.8% with rhodopsin.

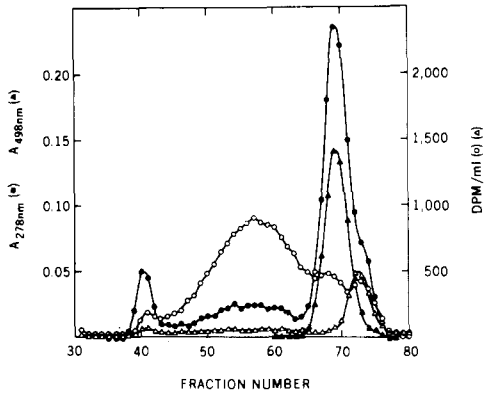

FIGURE 2: Agarose chromatography of extracts of rod outer segments from retinas incubated for 3 h with [^{3}H]mannose. Conditions were as outlined in Figure 1. Absorbance at 278 nm (●) and 498 nm (▲) of the control outer segment extract is plotted along with disintegrations min^{-1} ml^{-1} for [^{3}H]mannose incorporated in the presence (△) and absence (○) of 10^{-4} M puromycin. The amount of rhodopsin in the outer segments from the puromycin incubation was 138% of the control.

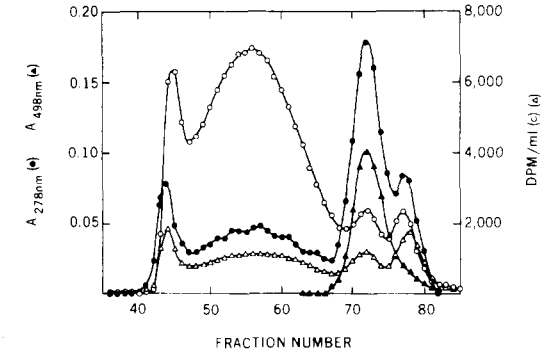

FIGURE 3: Agarose chromatography of extracts of rod outer segments from retinas incubated for 2 h with [^{3}H]glucosamine. Conditions were as outlined in Figure 1. Absorbance at 278 nm (●) and 498 nm (▲) of the outer segment extract from the puromycin incubation is plotted along with disintegrations min^{-1} ml^{-1} for [^{3}H]glucosamine incorporated in the presence (△) and absence (○) of 10^{-4} M puromycin. The amount of rhodopsin in the control outer segments was 121% of that from the puromycin experiment.

effectively blocked leucine incorporation into both rhodopsin and opsin as isolated in the rod outer segments. Labeling with mannose was almost as effectively blocked by puromycin. The specific activity of opsin is a more accurate measure of the effect since the rhodopsin specific activity is affected by the radioactive peak that immediately trails rhodopsin. A small amount of mannose appeared in opsin (3.2 to 5% of the control compared with 1.1 to 1.6% of the control with leucine) possibly reflecting the existence of a very small pool of newly synthesized opsin lacking an oligosaccharide. Such a pool could act as an acceptor for mannose even in the absence of new polypeptide synthesis.

Glucosamine incorporation in the presence of puromycin appeared not to continue after 2 h since the specific activity of neither opsin nor rhodopsin showed any change between 2 and 4 h. These results would be consistent with the labeling of a pool of preexisting opsin, possibly in the Golgi complex. Once that pool had been labeled, further incorporation would have ceased in the absence of protein synthesis. It should be noted that the proportions of labeled rhodopsin and opsin were not the same in the control and experimental outer segment extracts (Figure 3). The higher proportion of labeled rhodopsin in the puromycin treated retina may be the result of the fact that the pigment epithelium, the source of 11-*cis*-retinal, the chromophore of rhodopsin, was not present during the incubation of the retinas. As a result, the availability of the chromophore quickly became limiting. The shorter time of actual

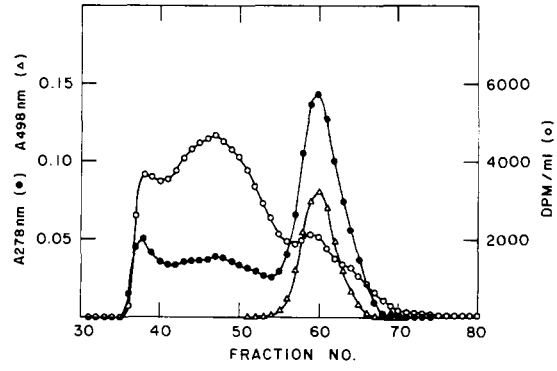

FIGURE 4: Agarose chromatography of an extract of rod outer segments from retinas incubated with [^{3}H]glucosamine. Conditions were as outlined in Figure 1 except that the detergent used for elution was Emulphogene. Absorbance at 278 nm (●) and 498 nm (△) is plotted along with disintegrations min^{-1} ml^{-1} (○).

labeling in the presence of puromycin reflects the initial availability of chromophore, hence the higher proportion of rhodopsin.

Since glucosamine labeling of opsin and rhodopsin was not totally sensitive to puromycin, the identity of the major labeled product, opsin, deserves demonstration. Glucosamine labeled outer segments were prepared as before and equal quantities were extracted and chromatographed without additional treatment (Figure 4) or after incubation with 9-*cis*-retinal

TABLE II: Effect of Puromycin on Galactose Incorporation.

Precursor(s)	Sp Act.[a] Rhodopsin	Sp Act.[a] Opsin
[^{3}H]Galactose		
Control	17 417 ± 199	35 826 ± 74
+ Puromycin	20 267 ± 221	52 147 ± 98
[^{3}H]Galactose/[^{14}C]Mannose		
Control	15 939 ± 212/(675 ± 50)	35 894 ± 47/(2006 ± 18)
+ Puromycin	12 642 ± 211/(232 ± 43)	27 138 ± 41/(302 ± 8)

[a] Specific activity is defined as dpm per ml per A_{498nm} for rhodopsin and dpm per ml per A_{278nm} for opsin. Retinas were incubated for 3 h. The standard deviation is shown for each dpm determination.

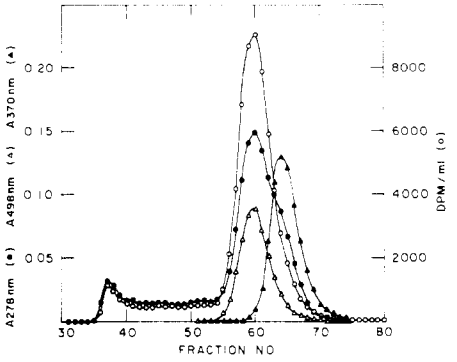

FIGURE 5: Agarose chromatography of an extract of [^{3}H]glucosamine labeled rod outer segments which had been incubated with 9-*cis*-retinal. Conditions were as in Figure 4. Absorbance of visual pigment at 278 nm (●) and 498 nm (▲) and of 9-*cis*-retinal at 370 nm (▲) is plotted along with disintegrations min^{-1} ml^{-1} (○).

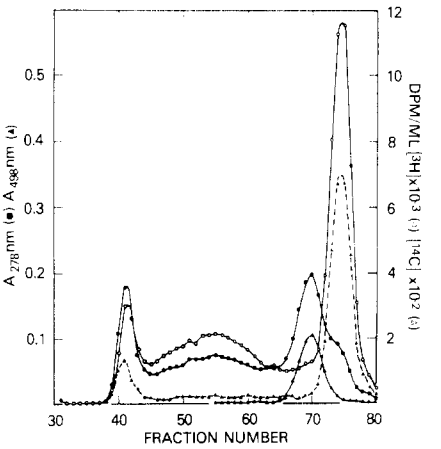

FIGURE 6: Agarose chromatography of extracts of rod outer segments from retinas incubated for 3 h with [^{3}H]galactose and [^{14}C]mannose in the presence of 10^{-4} M puromycin. Conditions were as outlined in Figure 1. Absorbance at 278 nm (●) and 498 nm (▲) is plotted along with disintegrations min^{-1} ml^{-1} for [^{3}H]galactose (○) and [^{14}C]mannose (△).

(Figure 5). It can be seen that the 9-*cis*-retinal treatment caused the large opsin peak (Figure 4) to be converted to visual pigment (Figure 5) with very little radioactivity remaining in the opsin region. Consequently, the glucosamine labeled product migrating as opsin was almost entirely opsin since it combined with the chromophore to produce labeled visual pigment (O'Brien and Muellenberg, 1975).

Effect of Puromycin on Galactose Incorporation. Since galactose is incorporated into rhodopsin by isolated bovine retinas (O'Brien, 1976), the effect of puromycin was studied in an attempt to determine the location of galactose transfer. Incubations of groups of four retinas were carried out as before using [^{3}H]galactose as the labeled precursor. In some experiments [^{14}C]mannose was added along with [^{3}H]galactose to provide an alternate internal monitor of the effect of puromycin. Agarose chromatography of control outer segment extracts revealed the expected labeling pattern for [^{14}C]mannose and a similar pattern for [^{3}H]galactose as previously observed (O'Brien, 1976). However, 10^{-4} M puromycin did not block the incorporation of [^{3}H]galactose even though the incorporation of [^{14}C]mannose was abolished in a 3-h incubation (Figure 6). (The quantity of radioactivity found in the peak trailing rhodopsin varies greatly and is correlated with radioactivity and 278-nm absorbance at the void volume, suggesting variable amounts of a contaminating membrane fraction in the outer segment preparations. This material influences the specific activity determinations for rhodopsin but not for opsin since treatment of galactose- or mannose-labeled outer segments prior to extraction caused all the radioactivity migrating as opsin to be converted to visual pigment (O'Brien, 1976, 1977)). The results of both single and double label experiments are summarized in Table II. It can be seen that puromycin produced no consistent effect on galactose incorporation, causing a stimulation in one experiment and a slight inhibition in the other. This anomaly is probably a reflection of the variable extent of galactose incorporation found in other experiments where, in duplicate incubations, the specific activity of rhodopsin differed by 14 to 32%. It is clear, however, in the double-label experiment that puromycin had substantially blocked mannose incorporation, and yet galactose incorporation continued essentially as in the control. Thus galactose does not appear to be incorporated into newly-synthesized rhodopsin or opsin. The location of galactose incorporation might possibly be the rod outer segment.

Discussion

Puromycin prevents leucine labeling of rhodopsin and opsin as isolated in the rod outer segments, the photoreceptor organelles that contain the visual pigment. Some labeling could occur in the inner segment rough endoplasmic reticulum but, if it does, the labeled proteins are unable to migrate to the outer segment without continued protein synthesis. The nearly total abolition of mannose incorporation by puromycin is consistent with the idea that lipid intermediates play a role in core oligosaccharide synthesis as found in several glycoprotein synthetic systems (Lennarz, 1975). Through this mechanism, an oligosaccharide of mannose and *N*-acetylglucosamine would be initially assembled as a lipid-bound complex. The completed

oligosaccharide would then be transferred to a newly synthesized polypeptide, perhaps even as it is still in the process of elongation (Cowan and Robinson, 1970; Lawford and Schachter, 1966; Molnar et al., 1965; Molnar and Sy, 1967). Recently, Kean and Plantner (1976) synthesized lipid-bound oligosaccharides in bovine retina homogenates using labeled GDP mannose as a donor. They also found a small amount of labeled visual pigment among the products. Thus, their results support the idea that the lipid intermediate mechanism is involved in the synthesis of the core oligosaccharide of rhodopsin in the retina but that there is at best a very small pool of nascent opsin capable of acting as an acceptor for mannose residues. In the present experiments, labeling of opsin with mannose in the inner segment would not be detectable, as mentioned above in reference to leucine, if the proteins were unable to continue migrating to the outer segment in the absence of continued protein synthesis as a result of puromycin inhibition.

The inability of puromycin to produce a similar blockage of glucosamine incorporation is a clear indication that not all the glucosamine residues in rhodopsin are truly part of a core oligosaccharide as defined above. On the contrary, Heller and Lawrence (1970) presented evidence that some glucosamine could be removed by enzyme digestion of glycopeptides derived from rhodopsin, leaving all the mannose residues and some glucosamine, presumably the residue(s) linked to asparagine, still bound to the peptide. This peripheral glucosamine could be the first residue of a terminal trisaccharide, like that of thyroglobulin, which is thought to be added to the core oligosaccharide in the Golgi complex of the thyroid (Chabaud et al., 1974). Spiro and Spiro (1966) using thyroid slices found that puromycin inhibited mannose incorporation into particle-bound thyroglobulin almost as effectively as it did leucine incorporation. The incorporation of glucosamine, the other core sugar, was not so effectively blocked, however, just as in the present experiments. A pool of preformed polypeptides in the thyroid Golgi complex probably served as an acceptor for glucosamine incorporation for a time after inhibition of protein synthesis by puromycin. The puromycin-insensitive glucosamine incorporation apparently represented the synthesis in the thyroid Golgi complex of the first glycosidic bond of the terminal trisaccharide which is built up by the sequential transfer of *N*-acetylglucosamine, galactose, and fucose (or sialic acid) to the core oligosaccharide (Spiro and Spiro, 1968). The experiments reported here can be interpreted in the same way.

Autoradiographic evidence (Bok et al., 1974) indicates that there is incorporation of glucosamine, possibly into rhodopsin, in the Golgi complex of frog photoreceptors. This evidence, therefore, also suggests that a terminal trisaccharide is initiated in the photoreceptor Golgi complex. But the transfer of galactose, the second residue of the trisaccharide, does not appear to occur in the Golgi complex. Puromycin should have a similar effect on the incorporation of any sugar in the Golgi complex. Yet glucosamine incorporation was blocked after an initial incorporation, whereas galactose incorporation was essentially unaffected by puromycin. Furthermore, galactose is not normally detected as a component of purified rhodopsin (Heller and Lawrence, 1970; Plantner and Kean, 1976). Recent evidence that galactose and fucose can be transferred to rhodopsin from sugar nucleotides by rod outer segment preparations (O'Brien, 1976) suggests that a terminal trisaccharide, if it is ever assembled in vivo, may not be assembled totally in the Golgi complex. Only the first residue of *N*-acetylglucosamine seems to be added there, with galactose and fucose probably being added in the outer segment, perhaps as a preparation of the tips of the outer segments for shedding and phagocytosis by the pigment epithelium, the layer of cells adjacent to the photoreceptors (Young and Bok, 1969). Since only the tips of the outer segments are shed, only a small fraction of the rhodopsin might be expected to possess galactose residues which could easily escape detection. The function of a terminal trisaccharide might be to create a new recognition site enabling the pigment epithelium cells to distinguish the shed packets of rod outer segment membranes from the intact, functional outer segment.

The present experiments can also be interpreted as showing the possible transfer of *N*-acetylglucosamine residues to opsin and rhodopsin in the rod outer segment as well as in the Golgi complex. These alternatives cannot be adequately differentiated by the data. Likewise, the puromycin-insensitive incorporation of all four precursors into the peak following rhodopsin cannot be explained at present. Several possibilities exist such as mitochondrial or synaptosomal protein or oligosaccharide–lipid synthesis or a combination of these.

Acknowledgments

Many helpful discussions with Dr. Dean Bok are gratefully acknowledged as is the technical assistance of Mrs. Consuelo G. Muellenberg.

References

Basinger, S. F., and Hall, M. O. (1973), *Biochemistry 12,* 1996.

Bok, D., Basinger, S. F., and Hall, M. O. (1974), *Exp. Eye Res. 18,* 225.

Bouchilloux, S., Chabaud, O., Michel-Béchet, M., Ferrand, M., and Athouël-Haon, A. M. (1970), *Biochem. Biophys. Res. Commun. 40,* 314.

Chabaud, O., Bouchilloux, S., Ronin, C., and Ferrand, M. (1974), *Biochimie 56,* 119.

Cowan, N. J., and Robinson, G. B. (1970), *FEBS Lett. 8,* 6.

Heller, J. (1968), *Biochemistry 7,* 2906.

Heller, J., and Lawrence, M. A. (1970), *Biochemistry 9,* 864.

Kean, E. L., and Plantner, J. J. (1976), *Exp. Eye Res. 23,* 89.

Lawford, G. R., and Schachter, H. (1966), *J. Biol. Chem. 241,* 5408.

Lennarz, W. J. (1975), *Science 188,* 986.

McQuillan, M. T., and Trikojus, V. M. (1972), in Glycoproteins: Their Composition, Structure and Function, second ed, Gottschalk, A., Ed., New York, N.Y., Elsevier, p 926.

Molnar, J., Robinson, G. B., and Winzler, R. J. (1965), *J. Biol. Chem. 240,* 1882.

Molnar, J., and Sy, D. (1967), *Biochemistry 6,* 1941.

Montgomery, R. (1972), in Glycoproteins: Their Composition, Structure and Function, second ed, Gottschalk, A., Ed., New York, N.Y., Elsevier, p 518.

O'Brien, P. J. (1976), *Exp. Eye Res. 23,* 127.

O'Brien, P. J. (1977), *Exp. Eye Res.* (in press).

O'Brien, P. J., and Muellenberg, C. G. (1974), *Exp. Eye Res. 18,* 241.

O'Brien, P. J., and Muellenberg, C. G. (1975), *Biochemistry 14,* 1695.

O'Brien, P. J., Muellenberg, C. G., and Bungenberg de Jong, J. J. (1972), *Biochemistry 11,* 64.

Papermaster, D. S., and Dreyer, W. J. (1974), *Biochemistry 13,* 2438.

Plantner, J. J., and Kean, E. L. (1976), *J. Biol. Chem. 251,*

1548.
Shichi, H., Lewis, M. S., Irrevere, F., and Stone, A. (1969), *J. Biol. Chem. 244*, 529.
Spiro, M. J., and Spiro, R. G. (1968), *J. Biol. Chem. 243*, 6529.
Spiro, R. G., and Spiro, M. J. (1966), *J. Biol. Chem. 241*, 1271.
Young, R. W., and Bok, D. (1969), *J. Cell Biol. 42*, 392.

Diversity of Sequences of Polyadenylated Cytoplasmic RNA from Rainbow Trout (*Salmo gairdnerii*) Testis and Liver†

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ABSTRACT: We have compared the sequence complexity and diversity of polyadenylated cytoplasmic RNA derived from two differentiated trout tissues: liver and testis. The kinetics of hybridization of polyadenylated RNA from each of these tissues with complementary DNA synthesized by reverse transcriptase revealed three abundance classes for liver RNA, the first comprising 4 sequences, the second 120, and the third, 20 000; in contrast, testis RNA showed only two abundance classes containing 6 and 6100 different RNA sequences, respectively, and of average length 6×10^5 daltons. The extent of overlapping among those two RNA populations was further studied by performing heterologous annealing reactions between cDNA and a vast excess of mRNA. Liver mRNA was complementary to 80% of the testis cDNA. Conversely, testis mRNA reacted with only 25% of the liver cDNA. Experiments with fractionated cDNA probes indicated that the unshared sequences belonged mainly to the less frequent, most complex, class of mRNAs.

The mechanisms by which gene expression is regulated in eukaryotes remain unclear. The active genes in a given tissue may be divided into two sets: those genes required for functions common to all cells of the organism, e.g., the common enzymes of metabolism, membrane components, etc., which may be termed "housekeeping genes", and those which determine the specialized functions of the cell. The relative sizes of these two sets of genes could vary widely so that, at one extreme, the set of "housekeeping genes" would be large in relation to those for specialized functions, while, at the other, this set could be small in relation to the set of specialized genes (Galau et al., 1976).

One approach to this problem involves a comparative study of the messenger RNA populations of different specialized tissues. Two experimental procedures are currently available for the study of sequence complexity of RNA populations. In the first, hybridization reactions are allowed to take place to saturation between radioactive unique sequence DNA and RNA (Hahn and Laird, 1971; Levy W. et al., 1976a,b). The amount of DNA in hybrid form at saturation gives information about the extent of transcription from this particular class of DNA. The second procedure involves synthesis of highly radioactive DNA copies of polyadenylated RNA fractions by reverse transcriptase in the presence of oligo(dT) primers and hybridization of the cDNA to a vast excess of RNA template (Bishop et al., 1974; Birnie et al., 1974; Ryffel and McCarthy, 1975; Levy W. and McCarthy, 1975; Axel et al., 1976). The kinetics of hybridization allows not only a measurement of the number of different sequences present as mRNA, but also gives information regarding the relative abundance of these sequences within the RNA population. This method has the additional advantage of dealing with transcripts from both repetitive and unique DNA.

In this report, we have used the second procedure to analyze the diversity of sequences of polyadenylated RNA from two specialized tissues of the trout: liver and testis.

Materials and Methods

Preparation of Liver and Testis Cytoplasmic RNA. Livers were removed from rainbow trout (*Salmo gairdnerii*), maintained in the laboratory aquarium at 12–13 °C, and stored frozen at −70 °C for short periods of time before utilization.

Testis were collected at a late stage of maturation (in October 1973) from freshly killed trout (Dantrout, Brande, Denmark), immediately frozen on dry ice, and stored frozen at −70 °C.

For a typical RNA preparation, 20 g of frozen tissue (liver or testis) was broken into small pieces, and then allowed to thaw at room temperature for about 10 min. Two volumes of a solution of TMKS buffer (0.25 M sucrose, 40 mM Tris[1]-HCl, pH 7.4, 25 mM KCl, and 5 mM $MgCl_2$) were added and the tissue was first homogenized (at 4 °C) in a mortar and then in a glass homogenizer with a Teflon pestle at a slow speed (three to four strokes). The homogenate was filtered through four layers of gauze and subsequently centrifuged at 800*g* for 7 min at 4 °C. The nuclear pellet was kept for the isolation of DNA.

The cytoplasmic supernatant was adjusted to 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5% sodium dodecyl sulfate, and the RNA was extracted with phenol-chloroform and chloroform–isoamyl alcohol as described

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[1] Abbreviations used are: mRNA, polyadenylated cytoplasmic RNA; cDNA, complementary DNA; poly(A), polyadenylic acid; rRNA, ribosomal RNA; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.