Vol. 112, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1983 Pages 115-125

BUTYRATE REGULATES GLYCOSYLATION OF THE GLYCOPROTEIN HORMONE ALPHA SUBUNIT SECRETED BY "GLUCOSE-STARVED" HUMAN LIVER CELLS*

Jay S. Morrow†, Bruce D. Weintraub and Saul W. Rosen

Clinical Endocrinology Branch
National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases
National Institutes of Health, Bethesda, Maryland 20205

Received February 23, 1983

SUMMARY: The α-subunit secreted by Chang human liver cells was labeled in vitro with [^{35}S]Met, [^{3}H]GlcN, or [^{3}H]Fuc, and the biosynthetic products were studied by SDS-PAGE. Cells labeled with [^{35}S]Met secreted a homogeneous 23K-24K α. In contrast, α secreted from cells labeled with [^{3}H]GlcN and [^{3}H]Fuc in the absence of glucose ("glucose-starved") was heterogeneous. This size heterogeneity was altered by glucose and by butyrate, but was little affected by dibutyryl adenosine 3',5'-monophosphate. In the presence of 0.56 mM (10 mg/dl) or 5.6 mM (100 mg/dl) glucose, or 2 mM butyrate, primarily the larger and presumably more highly glycosylated 24K-25K α was secreted. Moreover, the effect of 2 mM butyrate on the α secreted by "glucose-starved" cells was qualitatively and quantitatively similar to the effect of 0.56 mM glucose in the absence of butyrate. Likewise, 2 mM butyrate + 0.56 mM glucose was nearly equivalent to 5.6 mM glucose alone. These results demonstrate a novel effect of butyrate on glycoprotein biosynthesis; it is the only agent, reported thus far, which has the same effects as Glc or Man on protein glycosylation in "glucose-starved" cells.

The glycoprotein hormones, CG‡, LH, FSH, and TSH, are composed of two dissimilar subunits, α and β, which are noncovalently linked to form the intact hormone (1). Within a species, the α-subunits are nearly identical, but the β-subunits are unique to each hormone. In addition to placenta and trophoblastic

*Presented in part at the 64th Annual Meeting of The Endocrine Society, San Francisco, CA, 1982.

†Present address: Division of Metabolism and Endocrine Drug Products, Food and Drug Administration, 5600 Fishers Lane, Room 14B04, Rockville, MD 20857.

‡Abbreviations: α, α-subunit of the glycoprotein hormones; CG, chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; CG-β, β-subunit of chorionic gonadotropin; BU, butyrate; DBC, dibutyryl adenosine 3',5'-monophosphate; Glc, glucose; Man, mannose; Met, methionine; GlcN, glucosamine; Fuc, fucose; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; GlcNAc, N-acetylglucosamine; M_r, relative molecular weight; RIA, radioimmunoassay.

Address reprint requests to Dr. Rosen, Bldg. 10, Rm. 8N315.

0006-291X/83 $1.50

neoplasms, a variety of nontrophoblastic human cancers secrete CG, α, CG-β, or some combination of these, both in vivo and in vitro (2-7).

The Chang human liver cell line secretes α, but not CG or CG-β (8). BU and DBC affect the secretion of CG, CG-β, or α from different cell lines (9-12), but little is known about the effects of these agents on post-translational events such as glycosylation (13). Recently, it was shown that the absence of Glc ("Glc-starvation") has major effects on protein glycosylation which can be reversed by the addition to the culture medium of Glc or Man (14-18), but not fructose, galactose, pyruvate, glycerol, inositol, glutamine, glycine, or GlcNAc (14, 17). In the experiments reported here, we studied the effects of BU, DBC, and Glc on α secreted from Chang cells.

MATERIALS AND METHODS

Cell Cultures: Chang liver cells were maintained in RPMI 1640 medium (containing 11.1 mM [200 mg/dl] Glc) supplemented with 20% fetal bovine serum (Grand Island Biologicals), 25 mM HEPES, 25 units/ml penicillin, and 25 μg/ml streptomycin, in humidified 95% air-5% CO_2.

Radiolabeling: Cells were plated into 25-cm^2 plastic tissue culture flasks (Beckton Dickinson) containing 4 ml of the supplemented RPMI medium, and grown to subconfluence. Then medium was completely replaced with fresh medium either alone, or containing 2 mM BU, or 2 mM DBC. After 24 hr, the medium again was replaced completely with 1-2 ml of serum-free RPMI 1640 either alone, or with BU or DBC as above, also containing 25 μCi/ml of either [^{35}S]Met (Amersham, 1200 Ci/mmole), [^{3}H]GlcN (New England Nuclear [NEN], 31 Ci/mmole), or [^{3}H]Fuc (NEN, 56 Ci/mmole), and grown for an additional 24 hr. [^{35}S]Met labeling was performed in Met-free medium, and [^{3}H]-carbohydrate labeling was performed in Glc-free medium. In one experiment, labeling with [^{3}H]GlcN was performed in medium with Glc concentrations of 0, 0.56 mM (10 mg/dl), or 5.6 mM (100 mg/dl). After labeling, medium was removed, and cells were washed and lysed as described in (19). Lysates were analyzed for total protein by the method of Lowry (20); the medium was analyzed by immunoprecipitation of α followed by SDS-PAGE. Lysates were not analyzed by immunoprecipitation and SDS-PAGE because after a 24-hr incubation subunits are primarily in the medium (21).

Immunoprecipitation and SDS-PAGE: Immunoprecipitation was performed on 100 μl aliquots of medium using rabbit anti-α serum (final dilution 1:500), or non-immune rabbit serum. In some cases, antiserum directed against reduced and carboxymethylated α (anti-RCM-α) was used. The methods for these techniques and for gel slicing and liquid scintillation counting are described in (22). Counting efficiency was 61% for ^{35}S, and 33% for ^{3}H. Quantitative determinations of α were made by integrating the area under the curves in Figures 1-4.

RESULTS

Untreated Chang cells labeled with [^{35}S]Met secreted a homogeneous α with M_r 23K-24K, larger than the homogeneous 21K α (data not shown) derived from urinary CG (std-α) (Figure 1A). BU stimulated [^{35}S]Met incorporation into α

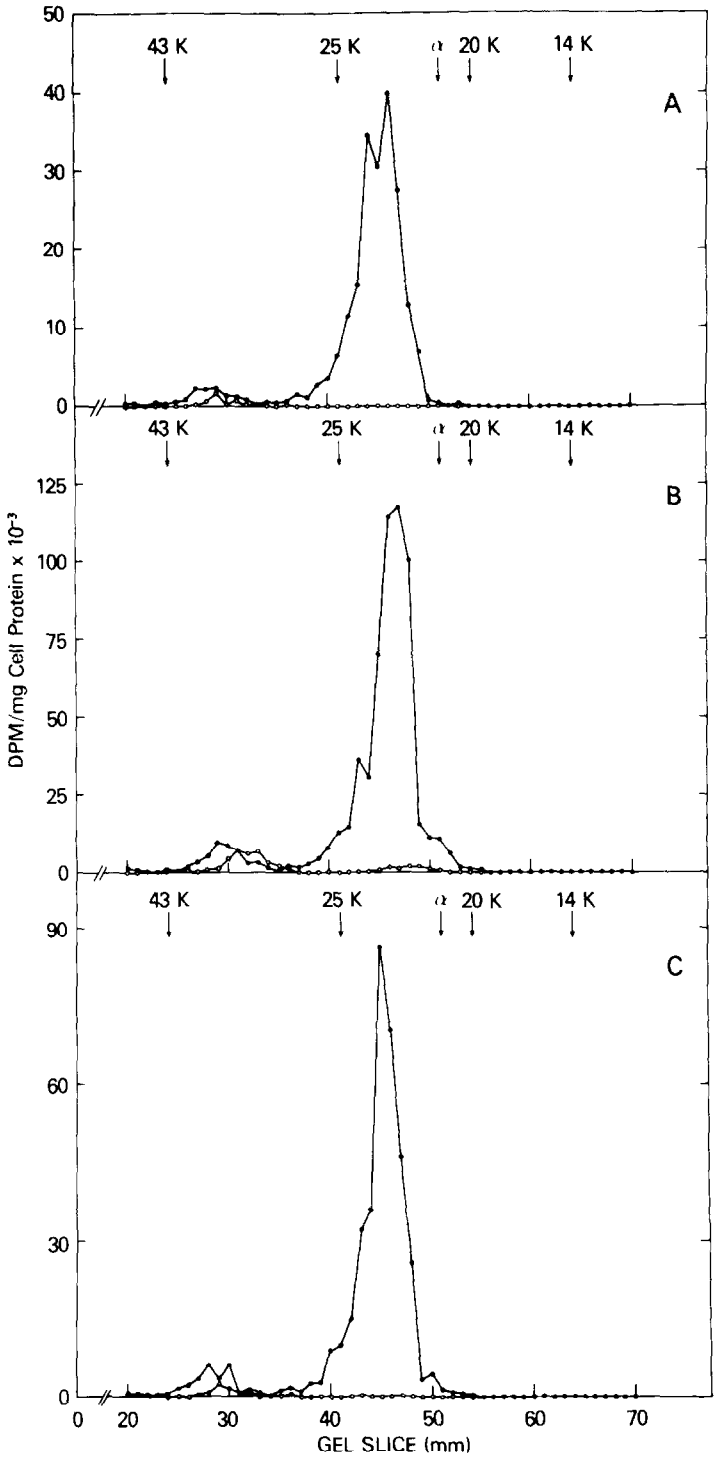


Figure 1: Effects of BU and of DBC on secreted α from Chang liver cells labeled for 24 hr with [^{35}S]Met. Shown are the results of SDS-PAGE of medium immunoprecipitated with anti-α serum (●) or nonimmune serum (○). Cells were grown in Met-free medium, untreated (A), treated with 2 mM BU (B), or treated with 2 mM DBC (C). Molecular weight markers are ovalbumin (43K), α-chymotrypsinogen (25K), soya bean trypsin inhibitor (20K), lysozyme (14K), and purified CR-119 std-α (α).

about 3-fold but did not change the migration of α on SDS-PAGE (Figure 1B). Similarly, DBC stimulated [^{35}S]Met incorporation into α about 1.8-fold, and also left its qualitative appearance on SDS-PAGE essentially unchanged (Figure 1C).

In contrast, α secreted from untreated cells labeled with [^{3}H]GlcN, in Glc-free medium, migrated on SDS-PAGE as two microheterogeneous peaks; 60% as a 24K-25K peak and 40% as a 19K-21K peak (Figure 2A). The dominant component of the latter co-migrated with std-α. BU stimulated [^{3}H]GlcN incorporation into total α about 4-fold, but the major effect was on the 24K-25K form. BU also induced a major qualitative change in α, with 86% migrating as a homogeneous 24K peak, and 14% as a homogeneous 22K peak (Figure 2B). Cells treated with DBC and labeled with [^{3}H]GlcN secreted a heterogeneous α, similar on SDS-PAGE to that secreted by untreated cells; in the presence of DBC, however, the smaller forms predominated, with 42% migrating between 24K-25K and 58% between 17K-21K. DBC had almost no effect on the quantitative incorporation of [^{3}H]GlcN into α (Figure 2C).

Untreated cells labeled with [^{3}H]Fuc in Glc-free medium also secreted an α that was heterogeneous on SDS-PAGE, with major peaks at 23K and 24K, and minor peaks at 20K and 25K (Figure 3A). BU stimulated [^{3}H]Fuc incorporation into α about 4-fold, and the secreted α now migrated as a dominant 24K peak, with a much smaller 22K shoulder (Figure 3B). DBC decreased [^{3}H]Fuc incorporation into α by 30%, and resulted in a broad microheterogeneous peak between 20K-24K (Figure 3C).

In most experiments, small amounts of material of M_r ~ 40K were immunoprecipitated by our anti-α serum, and even larger amounts were seen when immunoprecipitation was performed using anti-RCM-α (data not shown). This 40K material was larger than any species of CG-β we (22) or others (23, 24) have observed, and we were unable to detect CG-β in medium or lysates using a sensitive RIA (data not shown). Nevertheless, it could represent an aberrant form of CG-β which was originally combined with α, and hence was immunoprecipitated by our anti-α serum, but which was not recognized by the anti-CG-β serum used in our RIA. Other pos-

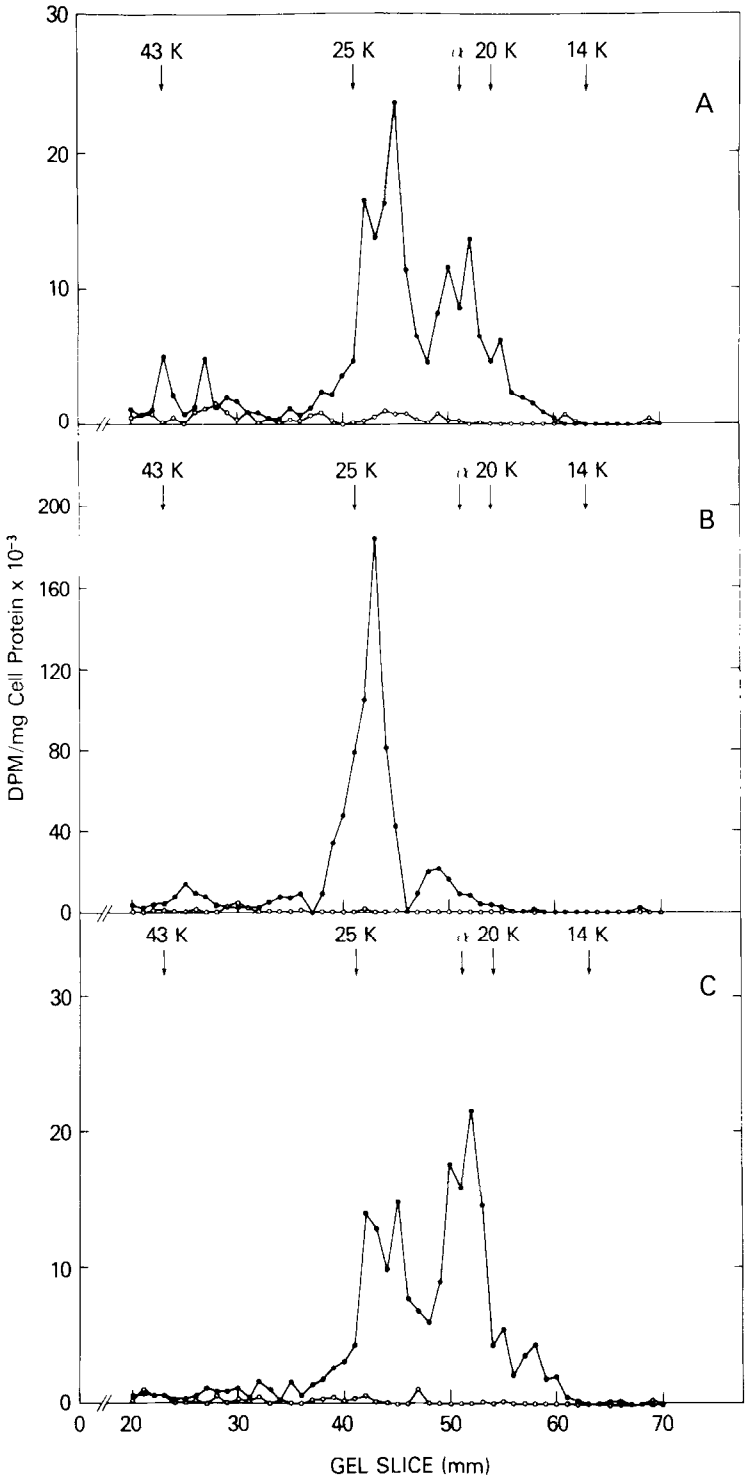


Figure 2: Same as Figure 1 except cells were grown in Glc-free medium and labeled with [^{3}H]GlcN.

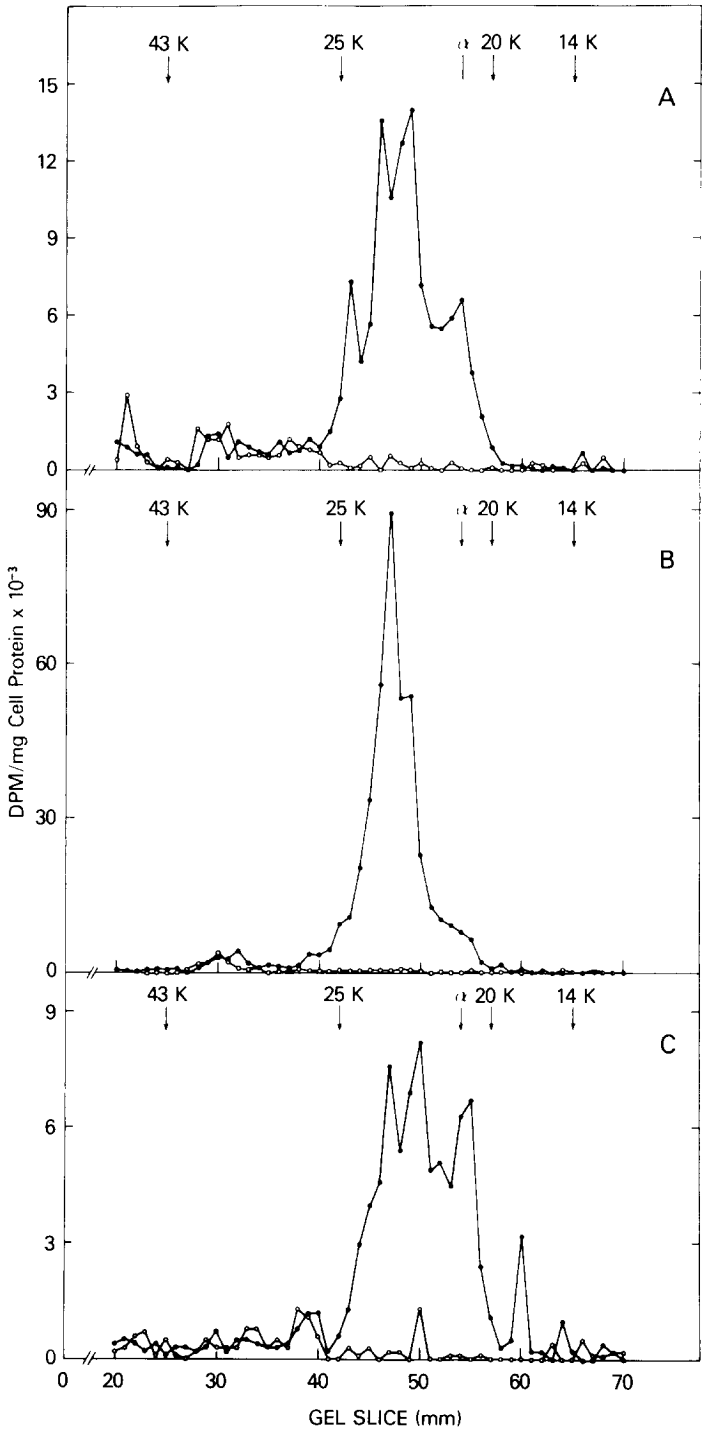


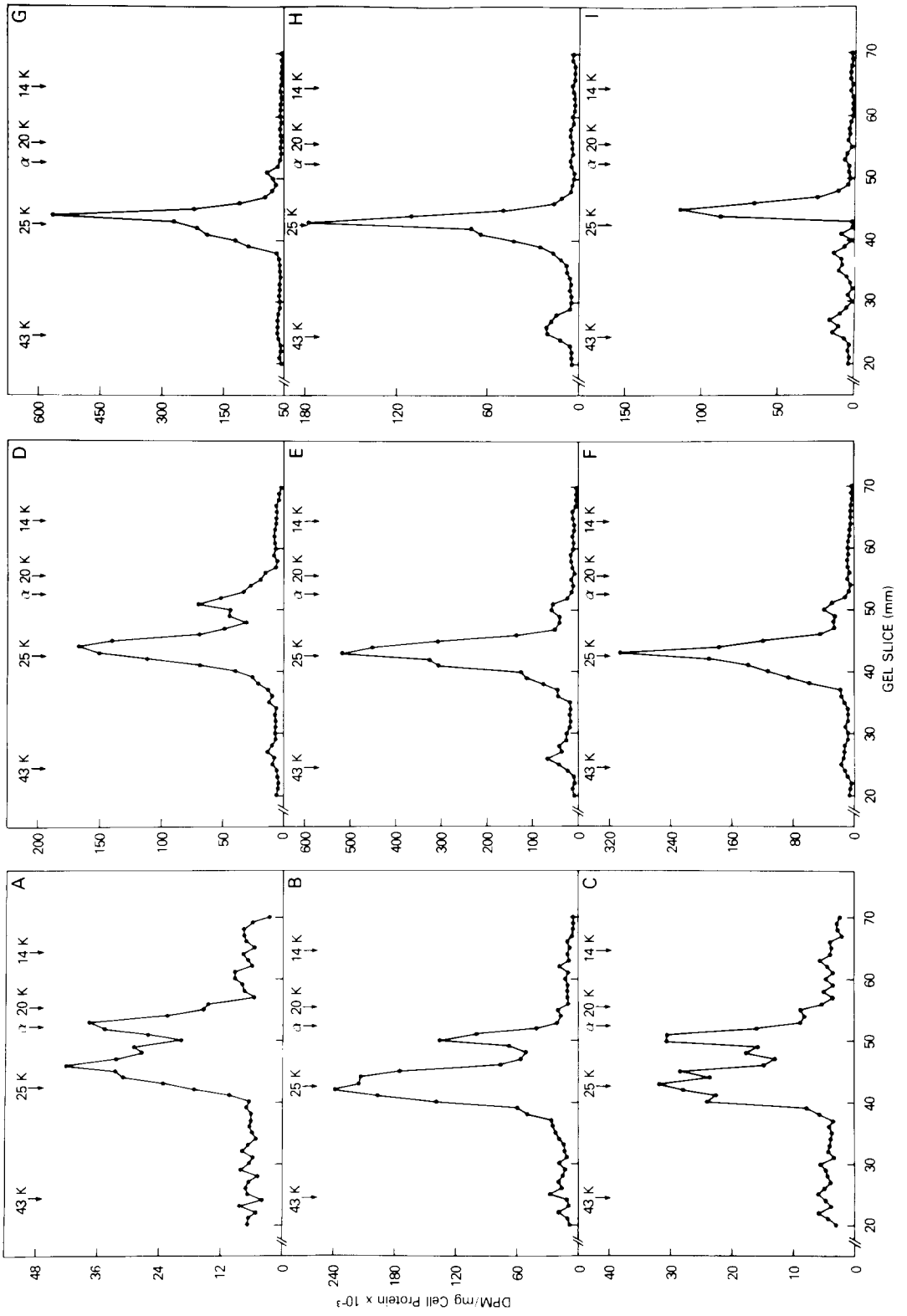
Figure 3: Same as Figure 1 except cells were grown in Glc-free medium and labeled with [^{3}H]Fuc.

sibilities include aggregates of α that were stable to the reducing and denaturing conditions used in SDS-PAGE (25), or some non-α species with epitopes recognized by our antisera.

We further investigated the effects of BU and DBC on secreted α by labeling with [^{3}H]GlcN in the presence of varying concentrations of Glc (Figure 4). Untreated cells labeled in Glc-free medium secreted 39% of total α as a 21K form and 61% as a 24K form (Figure 4A). When the ambient Glc was increased to 1/10 of the physiologic concentration (0.56 mM) the 24K α became even more dominant (Figure 4D); and when the Glc concentration was increased further to the physiologic range (5.6 mM) the 24K α was almost the sole species secreted (Figure 4G). The effect of BU on the α secreted by cells in the absence of Glc was qualitatively and quantitatively similar to 0.56 mM Glc in the absence of BU. Likewise, 2 mM BU + 0.56 mM Glc was nearly equivalent to 5.6 mM Glc alone (Figures 4A and 4B, 4D and 4E, or 4G and 4H). Furthermore, while BU stimulated [^{3}H]GlcN incorporation into α in the absence of Glc and in the presence of a low non-physiologic Glc concentration (0.56 mM), it markedly inhibited [^{3}H]GlcN incorporation at the physiological Glc concentration of 5.6 mM. In contrast, DBC in Glc-free medium did not substantially alter the size of secreted α (compare Figures 4C and 4A). However, at Glc concentrations of 0.56 mM and 5.6 mM, DBC did increase, albeit slightly, the secretion of the larger α (compare Figures 4F and 4D; 4I and 4C).

DISCUSSION

The large 23K-24K α seen with [^{35}S]Met labeling (Figure 1) is similar to that seen by us, and by many other workers, *in vitro* and *in vivo* (2, 5, 19, 22, 24). Studies of cell lines which secrete both complete CG and free α have shown two different forms of α on SDS-PAGE: a smaller one, 18K-20K, corresponding to the α which is combined with CG-β to form intact CG, and a larger one, 22K-24K, which is secreted as the free subunit (22-24, 26). Considerable evidence suggests that the larger M_r of free α is a result of increased glycosylation (27-29). Of interest in that regard is the presence of L-fucose in the free α secreted by Charg cells (Figure 3), which also is found on the α from JAR cells (26), and


A
B
C
D
E
F
G
H
I
43 K
25 K
α 20 K
14 K
DPM/mg Cell Protein x 10⁻³
GEL SLICE (mm)

JEG-3 choriocarcinoma cells (Jones-Brown, Rosen, and Weintraub, abstract submitted to 65th Annual Meeting of the Endocrine Society), but which is not found in std-α (30; Nilsson, Rosen, Weintraub, and Zopf, unpublished data). Similarly, in one study, the larger M_r of ectopic α secreted by HeLa cells compared to std-α was attributed to an increase in sialylation (28). In the DoT cervical carcinoma line, which secretes free CC-ß, the decreased binding of that ectopic subunit to concanavalin A is due to an extra GlcNAc linked to an inner Man (31). We predict, likewise, that the large ectopic α seen in our studies is the result of fucosylation, as well as increased amounts of sialic acid, and GlcN or its metabolites.

It is noteworthy that our finding of heterogeneity in ectopic α secreted by Chang liver cells labeled with [^{3}H]GlcN was not observed by others in eutopic α secreted by the JAR choriocarcinoma line, or in ectopic α secreted by HeLa or ChaGo cell lines (19). The reason for this difference is unclear, but may be related to differences in cell lines, antisera, or other methodologic factors.

The small ectopic α seen in these studies, in Glc-free medium, may have the same carbohydrate structure as std-α, but it seems more likely that it is different and presumably less glycosylated, and merely coincidentally co-migrates with std-α on SDS-PAGE. Thus, in other cell types "Glc-starvation" leads to intracellular accumulation of lipid-linked oligosaccharides of the structure $(Man)_5$-$(GlcNAc)_2$- or $(Glc)_3$-$(Man)_5$-$(GlcNAc)_2$- instead of the usual $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$- (16-18); and that the -$(Man)_5$- or other unusual species can be transferred to protein leading to failure of processing to "complex"-type oligosaccharides, resulting in abnormally glycosylated forms (16). Furthermore, abnormally (presumably less) glycosylated (15) and even nonglycosylated (14) forms of glycoproteins resulting from "Glc starvation" can be secreted.

The mechanism of the BU effect in this system is not known. BU increases histone acetylation and decreases histone phosphorylation in HeLa cells (32); such

---

Figure 4: Effects of BU, DBC, and Glc on secreted α from Chang liver cells labeled for 24 hr with [^{3}H]GlcN. Shown are the results of SDS-PAGE of media immunoprecipitated with anti-α serum. Cells were grown in Glc-free medium (A-C) or in medium containing 0.56 mM Glc (D-F), or 5.6 mM Glc (G-I), either untreated (A, D, G), treated with 2 mM BU (B, E, H), or treated with 2 mM DBC (C, F, I). Molecular weight markers are as in Figure 1.

post-translational modifications of histones may lead to transcription and ultimately translation of new proteins, such as glycosylating enzymes. Along this line, BU increases the activity of CMP-sialic acid:lactosylceramide sialyltransferase in HeLa cells (33). A BU-induced increase in glycosylating enzymes does not, however, explain our observation that BU inhibits the incorporation of [^{3}H]GlcN into α in the presence of physiologic Glc concentrations. An alternative hypothesis is that BU somehow increases the intracellular Glc pool. Then, when Glc availability is limited, BU increases incorporation of [^{3}H]GlcN; whereas when Glc is abundant, a further increase in its intracellular pool decreases incorporation of [^{3}H]GlcN, even though total α by RIA secreted into the medium increases (data not shown). In any event, BU is the only agent, reported thus far, which has the same effects as Glc or Man (14, 15, 17) on protein glycosylation in "Glc-starved" cells. These data raise the possibility that other metabolic or even endocrine factors regulate glycosylation.

ACKNOWLEDGEMENTS

We thank the National Hormone and Pituitary Program for providing purified CG-α, Dr. S. Birken for anti-RCM-α serum, Dr. S. Aaronson for providing the Chang liver cells, and Mrs. I. Calvert for technical assistance.

REFERENCES

1. Pierce, J. G. (1971) Endocrinology 89, 1331-1344.
2. Franchimont, P., Gaspard, U., Reuter, A., Heynen, G. (1972) Clin. Endocrinol. 1, 315-336.
3. Braunstein, G. D., Vaitukaitis, J. L., Carbone, P. P., Ross, G. T. (1973) Ann. Int. Med. 78, 39-45.
4. Weintraub, B. D., Rosen, S. W. (1973) J. Clin. Invest. 52, 3135-3142.
5. Rosen, S. W., Weintraub, B. D. (1974) N. Engl. J. Med. 290, 1441-1447.
6. Rosen, S. W., Weintraub, B. D., Vaitukaitis, J. L., Sussman, H. H., Hershman, J. H., Muggia, F. M. (1975) Ann. Int. Med. 82, 71-83.
7. Rosen, S. W., Weintraub, B. D., Aaronson, S. A. (1980) J. Clin. Endocrinol. Metab. 50, 834-841.
8. Chou, J. Y. (1978) In Vitro 14, 775-778.
9. Ghosh, N. K., Rukenstein, A., Cox, R. P. (1977) Biochem. J. 166, 265-274.
10. Chou, J. Y., Robinson, J. C., Wang, S. S. (1977) Nature 268, 543-544.
11. Hussa, R. O., Pattillo, R. A., Ruckert, A. C. F., Scheuermann, K. W. (1978) J. Clin. Endocrinol. Metab. 46, 69-76.
12. Rosen, S. W., Calvert, I., Weintraub, B. D., Tseng, J. S., Rabson, A. S. (1980) Cancer Res. 40, 4325-4328.
13. Cox, G. S. (1981) Biochemistry 20, 4893-4900.
14. Stark, N. J., Heath, E. C. (1979) Arch. Biochem. Biophys. 192, 599-609.
15. Turco, S. J. (1980) Arch. Biochem. Biophys. 205, 330-339.
16. Rearick, J. I., Chapman, A., Kornfeld, S. (1981) J. Biol. Chem. 256, 6255-6261.

17. Gershman, H., Robbins, P. W. (1981) J. Biol. Chem. 256, 7774-7780.
18. Turco, S. J., Pickard, J. L. (1982) J. Biol. Chem. 257, 8674-8679.
19. Ruddon, R. W., Hanson, C. A., Bryan, A. H., Putterman, G. J., White, E. L., Perini, F., Meade, K. S., Aldenderfer, P. H. (1980) J. Biol. Chem. 255, 1000-1007.
20. Lowry, O H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
21. Ruddon, R. W., Anderson, C., Meade, K. S., Aldenderfer, P. H., Neuwald, P. D. (1979) Cancer Res. 39, 3885-3892.
22. Dean, D. J., Weintraub, B. D., Rosen, S. W. (1980) Endocrinology 106, 849-858.
23. Ruddon, R. W., Hanson, C. A., Addison, N. J. (1979) Proc. Natl. Acad. Sci. USA 76, 5143-5147.
24. Benveniste, R., Conway, M. C., Puett, D., Rabinowitz, D. (1979) J. Clin. Endocrinol. Metab. 48, 85-91.
25. Weintraub, B. D., Krauth, G., Rosen, S. W., Rabson, A. S. (1975) J. Clin. Invest. 56, 1043-1052.
26. Ruddon, R. W., Bryan, A. H., Hanson, C. A., Perini, F., Ceccorulli, L. M., Peters, B. P. (1981) J. Biol. Chem. 256, 5189-5196.
27. Fein, H. G., Rosen, S. W., Weintraub, B. D. (1980) J. Clin. Endocrinol. Metab. 50, 1111-1120.
28. Cox, G. S. (1981) Biochem. Biophys. Res. Commun. 98, 942-951.
29. Cox, G. S. (1981) Cancer Res. 41, 3087-3094.
30. Kessler, M. J., Reddy, M. S., Shah, R. H., Bahl, O. P. (1979) J. Biol. Chem. 254, 7901-7908.
31. Cole, L. A., Hussa, R. O. (1981) Endocrinology 109, 2276-2278.
32. Boffa, L. C., Gruss, R. J., Allfrey, V. G. (1981) J. Biol. Chem. 256, 9612-9621.
33. Fishman, P. H., Simmons, J. L., Brady, R. O., Freese, E. (1974) Biochem. Biophys. Res. Commun. 59, 292-299.