Lysine-Rich Histone Phosphorylation. A Positive Correlation with Cell Replication†

Rod Balhorn, Roger Chalkley,* and Daryl Granner

ABSTRACT: We have compared the histones of a normal differentiated tissue (rat liver) and a cultured tumor cell line (hepatoma HTC cells) under conditions of either rapid cell replication (regenerating liver or hepatoma cells in exponential phase) or essentially no active cell division (normal liver or hepatoma cells in stationary phase). Significant electrophoretic differences between these various histones were only found for the lysine-rich (F_1) histones. We find that dividing cells (both normal and tumor) show extensive phosphorylation-induced electrophoretic microheterogeneity and that nondividing cells have essentially no phosphorylated lysine-rich histone species. The extent of F_1 phosphorylation in rapidly dividing HTC cells amounted to about 70% of the parental F_1 histone in midexponential growth. We have interpreted our results in terms of an involvement of histone phosphorylation with cell replication rather than as a device for modifying gene expression.

Individual histone molecules can be modified by methylation (Gershey *et al.*, 1968; Murray, 1964; Paik and Kim, 1967; Tidwell *et al.*, 1968), acetylation (Allfrey *et al.*, 1964; Candido and Dixon, 1971; Phillips, 1963; Shepherd *et al.*, 1971), or phosphorylation (Gutierrez and Hnilica, 1967; Ingles and Dixon, 1967; Kleinsmith *et al.*, 1966; Langan, 1969; Ord and Stocken, 1966; Sherod *et al.*, 1970; Sung *et al.*, 1971). Thus far, the biological meaning of these alterations has not been documented. Because of the complexity of the eucaryotic chromosome, it has proved difficult to conduct *in vitro* experiments devised to study the effects of histone modification upon the physicochemical properties of the chromosomal material.

To circumvent these difficulties, biologists have studied the occurrence of such changes as a function of the biologic state of a given cell. This has been exploited to give an insight into the role of modification by phosphorylation in two systems. (1) Dixon and his coworkers have observed an increase in phosphorylation (and acetylation) of the arginine-rich histone F_{2a1} of fish spermatids at a time when histones are replaced by protamines (Marushige *et al.*, 1969; Sung and Dixon, 1970). Presumably the role of phosphorylation in this system is to neutralize the positive charge of the histone so as to weaken its binding to DNA. (2) The most frequent reports of phosphorylation of somatic histones *in vivo* concern histone fractions F_1 and F_{2a2} in dividing cells (Gurley and Walters, 1971). A reliable assay for histone phosphorylation utilizes a high-resolution electrophoretic approach which detects the decrease in charge density of a parent histone as negatively charged phosphate groups are added. Using this approach, it has been demonstrated that a mouse ascites tumor cell system and regenerating rat liver show extensive phosphorylation of these two histone fractions, but that nondividing tissues such as liver do not contain detectable amounts of phosphorylated histone.

It was necessary to extend these initial observations in order to obtain further information concerning the relationship between phosphorylation of certain histone fractions and cell replication. We have, therefore, studied phosphorylation of histones in cultured hepatoma cells using a cell line which is still hormonally responsive, namely HTC cells (Granner *et al.*, 1968). We find that histone F_1 is extensively phosphorylated (~70%) during exponential growth and that this phosphorylation is largely abolished when the cells move into stationary phase. Since both nondividing liver and stationary-phase tumor cells show no phosphorylation, and regenerating liver and dividing tumor cells show considerable phosphorylation, we interpret our results in terms of an obligatory involvement of histone phosphorylation in DNA synthesis or cell replication rather than as a reflection of specific gene activation.

Materials and Methods

Cell Culture. HTC cells, derived from the ascites form of Morris hepatoma 7288C, were grown in suspension culture as described previously (Granner *et al.*, 1968). The medium used was Swim's 77 which was modified to contain 50 mM Tricine [*N*-tris(hydroxymethyl)methlyglycine, Calbiochem] and was supplemented with 5% fetal calf and 5% bovine serum (Grand Island Biological Co.). Cells were kept in exponential growth (2–8 × 10^5 cells/ml) except where specifically indicated. Cultures were grown in an atmosphere of air at 37°.

Partial Hepatectomies. Blue Spruce rats weighing 100–200 g were partially hepatectomized by the method of Higgins and Anderson (1931) as previously described (Balhorn *et al.*, 1971). Normal rat liver was obtained from rats of the same age and weight.

Histone Isolation. Whole histone was isolated from purified nuclei by the method of Panyim *et al.* (1971).

Lysine-rich histone (F_1) was extracted from whole histone by the method of Johns (1964) and converted to the sulfate by dialysis against 0.4 N sulfuric acid prior to precipitation with 95% ethanol.

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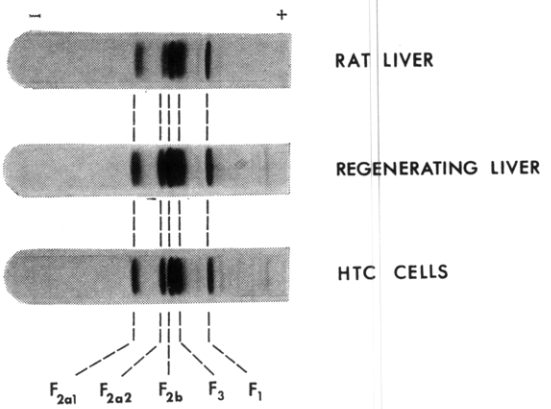


FIGURE 1: Polyacrylamide gel electrophoretic patterns of rat liver, 29-hr regenerating liver, and HTC histone. Electrophoresis was performed for 3.5 hr at 2 mA/gel and 130 V at 25°.

Electrophoresis. Electrophoresis of histone was performed at 4° on 25-cm gels (15% acrylamide) containing 2.5 M urea in 0.9 N acetic acid. After electrophoresis for 65 hr at 200 V, the gels were stained with Amido-Schwarz, destained, scanned on a Gilford microdensitometer, Model 2000, and the scans analyzed on a Dupont curve resolver as previously described (Balhorn *et al.*, 1971).

Hydrolysis of Histone Phosphate. The lysine-rich histones were treated with *Escherichia coli* alkaline phosphatase as previously described (Balhorn *et al.*, 1971).

Results and Discussion

Individual Histone Fractions. Electrophoretic patterns of whole histone from normal adult rat liver, regenerating liver, and exponentially growing hepatoma tissue culture cells (HTC cells) are shown in Figure 1. The histones are distinguished most by their apparent identity, even though the physiology of the parental tissues varies dramatically. The relative amounts and mobility of each of the five main histone groups are essentially constant no matter what the origin of the histone, an observation which is in general agreement with the observation of Johns (Johns *et al.*, 1970) who used a somewhat similar analytical system. The only detectable difference between histones of the three systems is seen in the appearance of a minor histone fraction (F_1^0), which is consistent with the observations of Panyim and Chalkley, who reported the presence of this histone fraction only in nondividing tissues (Panyim and Chalkley, 1969). During liver regeneration, this fraction (which is a lysine-rich histone) disappears at the onset of DNA synthesis and reappears when regeneration is complete.

Higher resolution of each fraction by means of electrophoresis on longer gels revealed that although the overall histone groups appear very similar, there were differences within the subfractions of the various groups. Differences in the microheterogeneity of histone groups F_{2a1} and F_1 are documented in Figure 2. About 50% of the F_{2a1} molecules in normal rat liver were in the acetylated, slower migrating form, whereas 40 and 25% were acetylated in regenerating liver and HTC cells, respectively. Thus, there appears to be no obvious correlation between the rate of cell replication and the overall extent of acetylation of this histone fraction, though these data give no information concerning relative rates of acetate uptake and turnover under the different physiological conditions described above.

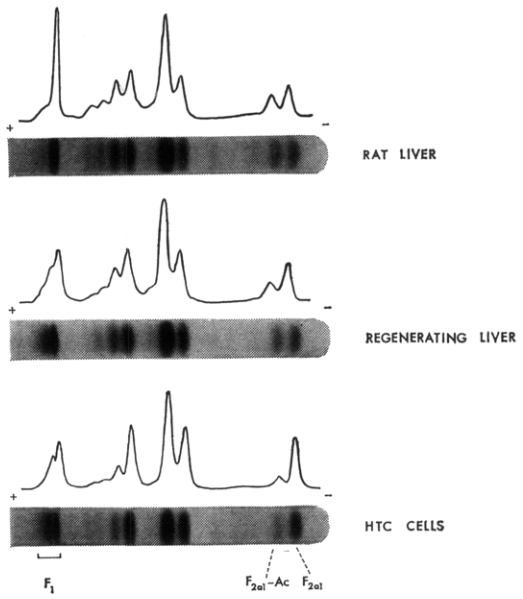


FIGURE 2: Long polyacrylamide gel electrophoretic patterns and microdensitometric scans of histone from rat liver, 29-hr regenerating liver, and HTC cells. Electrophoresis was performed at 2 mA/gel and 200 V for 40 hr at 0°.

The photographs and scans shown in Figure 2 also indicate that the heterogeneity of the lysine-rich (F_1) histone varies depending upon the tissue source. The resolution within this figure is not sufficient for a detailed analysis of this histone fraction and it was necessary to improve the resolving power of the system before this could be done (*vide infra*).

Electrophoretic Resolution of Lysine-Rich Histone Subfractions. Electrophoresis of the lysine-rich histone samples for 60–65 hr at 4° for 25 cm markedly enhances the resolution of the lysine-rich histone components, showing the presence of considerable microheterogeneity in all cases as seen in Figure 3. Normal rat liver F_1 consists of a major band (>90%) to-

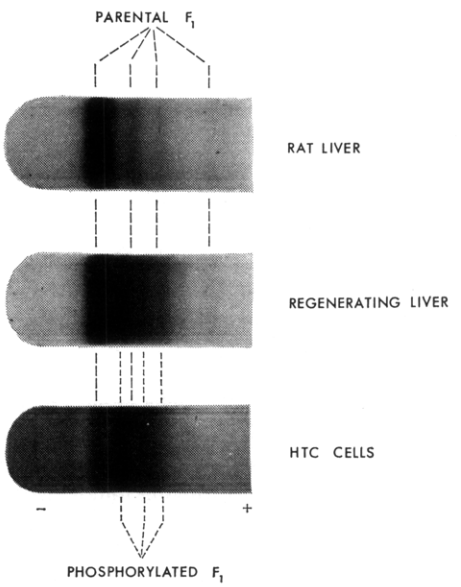


FIGURE 3: High-resolution gel electrophoretic patterns of the lysine-rich histones from rat liver, 29-hr regenerating liver, and HTC cells. Electrophoresis was performed at 2 mA/gel and 200 V for 65 hr at 0°.

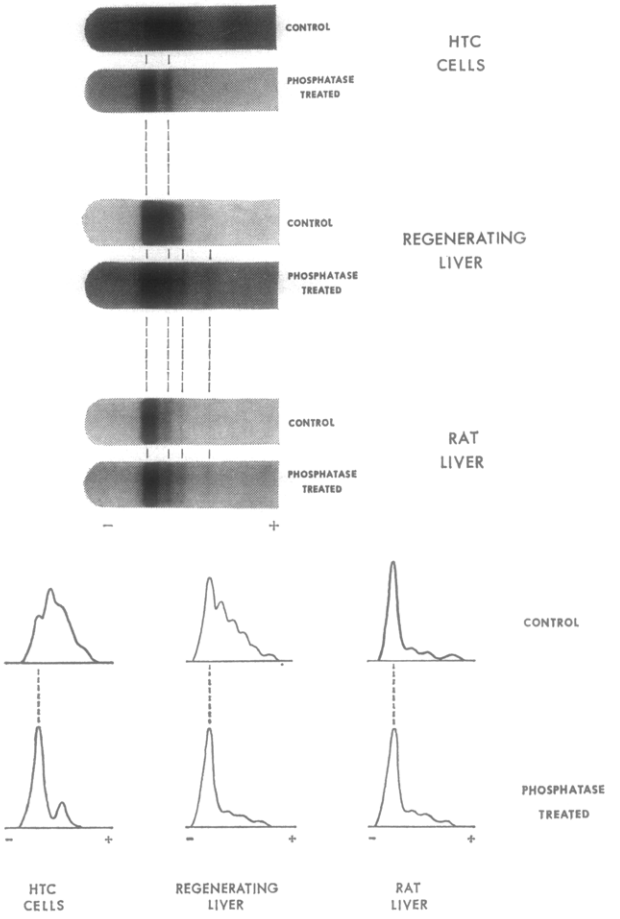
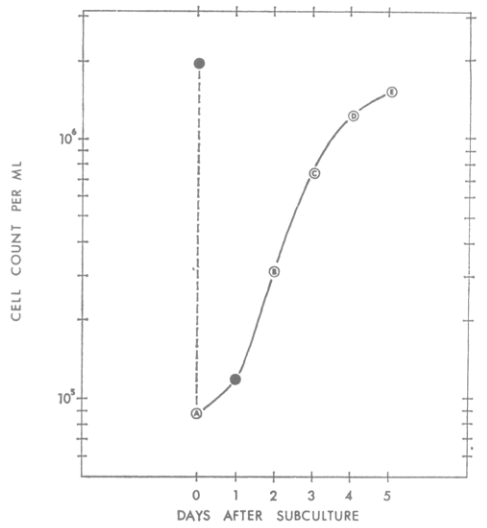


FIGURE 4: (a, top) High-resolution gel electrophoretic patterns of normal and phosphatase treated lysine-rich histones from adult rat liver, 29-hr regenerating liver, and exponentially growing HTC cells. (b, bottom) Microdensitometric scans of the gel electrophoretic patterns of normal and phosphatase-treated lysine-rich histones from the adult rat liver, regenerating liver, and exponentially growing HTC cells.

gether with three minor, slower bands. These bands probably represent F_1 histones of slightly different sequence. The spacing between the bands is irregular and it seems unlikely that they represent an electrophoretic heterogeneity due to differences in charge. Regenerating liver F_1 possesses two additional discrete bands in addition to all the bands characteristic of normal liver F_1. In contrast to liver, the fastest band contains only about 50% of the F_1 group; most of the rest of the material is found in the new bands moving more slowly than the parent. We have previously shown that these new bands seen in regenerating liver are phosphorylated forms of the fastest migrating parent F_1 histone (Balhorn *et al.*, 1971), though previously we had been unable to resolve the phosphorylated histone with anything like the clarity shown in Figure 3. The F_1 bands of HTC cells show the major liver parental band and two bands moving coincidentally with the phosphorylated F_1 histones described in regenerating liver. HTC cells lack the two slowest moving minor liver F_1 bands.

Demonstration of Phosphorylation of F_1 Histones. Confirmation of assignments as F_1 phosphohistones can be obtained as described previously (Sherod *et al.*, 1970; Balhorn *et al.*, 1971) by removing the phosphate group(s) with phosphatase and studying the attendant changes in mobility. The electrophoretic patterns and microdensitometric scans of the several F_1 histones following treatment with *E. coli* alkaline



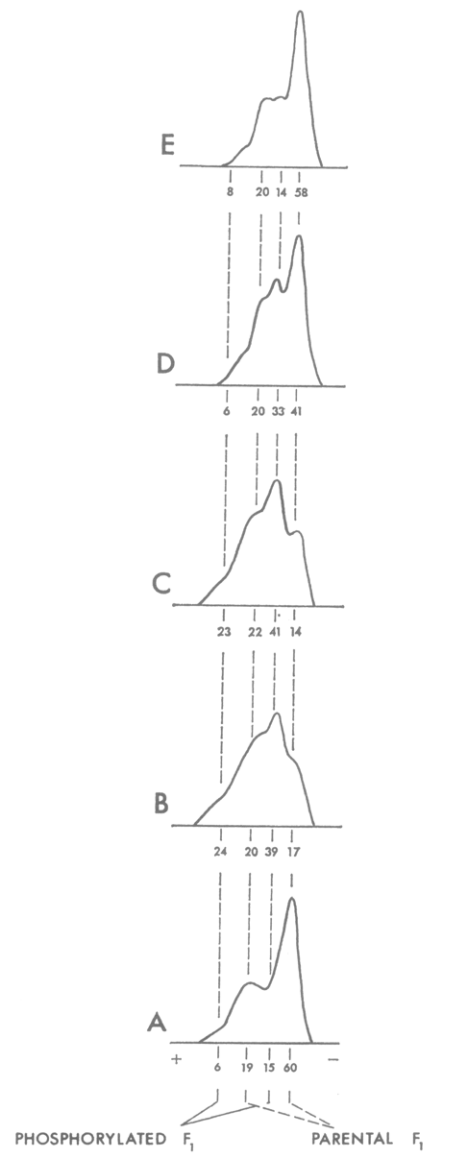


FIGURE 5: (a, left) Growth curve for HTC cells following subculture. Cells were subcultured from a stationary-phase culture to concentration A and samples taken at points B through E as described in Materials and Methods. (b, right) Microdensitometric scans of high-resolution electrophoretic patterns of lysine-rich histone from HTC cells samples A–E as taken from the growth curve. The relative percentage of each fraction determined by electronic curve resolution is shown below each scan.

phosphatase are shown in Figure 4a,b. Rat liver F_1 is totally unaffected by phosphatase treatment. However, substantial changes are observed in the F_1 histones of regenerating liver and HTC cells after hydrolysis and they now show patterns essentially identical with those of the control liver. Evidently the same parental F_1 fraction is being phosphorylated in both regenerating liver and HTC cells as the resulting phosphorylated molecules have the same electrophoretic mobility, though the extent of phosphorylation is greater in the cultured cells where about 70% of parent F_1 is phosphorylated.

The products from the phosphatase digestion confirm the notion that HTC cells do indeed lack two of the minor parent F_1 subfractions which are present in both adult rat liver and in regenerating rat liver. The distribution of F_1 histone between the two electrophoretic parent F_1 molecules is also different for HTC cells, showing the presence of slightly more of the slower moving fraction.

Visually we can identify two phosphorylated bands in HTC F_1 histone indicating a single and double level of phosphorylation (Balhorn *et al.*, 1972). Radioactivity studies indicate that an additional level of phosphorylation (three phosphate groups) is also present in small amounts.

Phosphorylation and Cell Replication in HTC Cells. We have previously argued that the extent of lysine-rich histone phosphorylation increases dramatically during the period of DNA synthesis and cell replication following partial hepatectomy; and further that following the cessation of cell replication, some 200 hr after partial hepatectomy, the phosphorylation of the F_1 histone was abolished. If this is a general phenomenon, one would predict that in cultured cells the transition from stationary to exponential growth, and *vice versa*, should be accompanied by appropriate changes in the level of phosphorylation. Suspension cultures of HTC cells in stationary (or resting) phase were subcultured into fresh media at a lower cell concentration and the cells harvested at several time intervals following subculture (Figure 5a). The lysine-rich histones were isolated and electrophoresed for 65 hr at 4° and microdensitometric scans of the resulting gels are shown in Figure 5b. Immediately following dilution, only a minimal amount (10–15%) of the lysine-rich histones were phosphorylated. During exponential growth, phosphorylation rapidly reaches a maximum at which time approximately 70% of the parent F_1 molecules are phosphorylated. As the cells leave exponential growth (96 hr), the degree of phosphorylation is dramatically reduced, and when they reach stationary phase, phosphorylation is again at a low level. The amount of material phosphorylated at the various times was calculated by curve analysis as described above and is included on the scans shown in Figure 5b.

Conclusions

The phosphorylation of mammalian lysine-rich F_1 histone appears to be a very accurate index of the involvement of the cell in replication. This holds true not only for a normal cell (liver) induced to divide for a finite time period during regeneration, but also for a liver tumor cell in both the exponential and stationary phase of growth. The nature of F_1 phosphorylation is very similar for both dividing systems which were both derived from rat liver. This is not surprising in view of the recent observations, both in this laboratory and in others (Stevely and Stocken, 1968; Balhorn *et al.*, 1971; R. Balhorn, M. Balhorn, and R. Chalkley, in preparation), which show a positive correlation between histone phosphorylation and cell replication. That the hepatoma cells should lose essentially all traces of phosphorylated F_1 when they reach stationary phase raises the question of the involvement of phosphorylation in molecular terms. It seems likely to us that it may be a necessary adjunct of cell replication and as such may play an important role in the control of this process. This notion is supported by recent observations from this laboratory which show that the F_1 and F_{2a2} histones are phosphorylated only at a time when DNA is synthesized (Balhorn *et al.*, 1972).

Since multiple phosphorylated F_1 histones have been noted in both the regenerating liver and exponentially growing HTC cells, it is possible that the quantitative aspects of phosphorylation may be an important regulatory mechanism as well. It is clear that we need to extend our understanding of the behavior of these several F_1 phosphohistones. Studies involved with the relative turnover rates of phosphate groups, as a function of the environmental status of the cell, are currently under way.

Acknowledgments

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References

Allfrey, V. G., Faulkner, R. and Mirsky, A. E. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 786.

Balhorn, R., Bordwell, J., Sellers, L., Granner, D., and Chalkley, R. (1972), *Biochem. Biophys. Res. Commun. 46*, 1326.

Balhorn, R., Rieke, W. O., and Chalkley, R. (1971), *Biochemistry 10*, 3952.

Candido, E. P. M., and Dixon, G. H. (1971), *J. Biol. Chem. 246*, 3182.

Gershey, E. L., Vidali, G., and Allfrey, V. G. (1968), *J. Biol. Chem. 243*, 5018.

Granner, D. K., Hayashi, S., Thompson, E. G., and Tomkins, G. M. (1968), *J. Mol. Biol. 35*, 291.

Gurley, L. R., and Walters, R. A. (1971), *Biochemistry 10*, 1588.

Gutierrez, R. M., and Hnilica, L. S. (1967), *Science 157*, 1324.

Higgins, G. M., and Anderson, R. M. (1931), *Arch Pathol. 12*, 186.

Ingles, C. J., and Dixon, G. H. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1011.

Johns, E. W. (1964), *Biochem. J. 92*, 55.

Johns, E. W., Davies, A. J. S., and Barton, M. E. (1970), *Biochim. Biophys. Acta 213*, 537.

Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1182.

Langan, T. A. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1276.

Marushige, K., Ling, V., and Dixon, G. H. (1969), *J. Biol. Chem. 244*, 5953.

Murray, K. (1964), *Biochemistry 3*, 10.

Ord, M. G., and Stocken, L. A. (1966), *Biochem. J. 98*, 888.

Paik, W. K., and Kim, S. (1967), *Biophys. Biochem. Res. Commun. 29*, 14.

Panyim, S., Bilek, D., and Chalkley, R. (1971), *J. Biol. Chem. 246*, 4206.

Panyim, S., and Chalkley, R. (1969), *Biophys. Biochem. Res. Commun. 37*, 1042.

Phillips, D. M. P. (1963), *Biochem. J. 87*, 258.

Shepherd, G. R., Noland, B. J., and Hardin, J. M. (1971), *Biochim. Biophys. Acta 228*, 544.

Sherod, D., Johnson, G., and Chalkley, R. (1970), *Biochemistry 9*, 4611.
Stevely, W. S., and Stocken, L. A. (1968), *Biochem. J. 110*, 187.
Sung, M. T., and Dixon, G. H. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1616.
Sung, M. T., Dixon, G. H., and Smithies, O. (1971), *J. Biol. Chem. 246*, 1358.
Tidwell, T., Allfrey, V. G., and Mirsky, A. E. (1968), *J. Biol. Chem. 243*, 707.

A Calorimetric Study of the Binding of Carbon Monoxide to Myoglobin†

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ABSTRACT: The heats of binding of carbon monoxide to both horse and sperm-whale myoglobin have been measured using a gas–liquid microcalorimeter. At pH 8.00 it was found that $\Delta H = -19.2$ kcal mole^{-1} for the reaction Mb(aq) + CO(g) → MbCO(aq) for horse Mb and $\Delta H = -23.9$ kcal mole^{-1} for sperm-whale Mb. Extensive dialysis before reaction changed these values to -18.1 and -20.7 kcal mole^{-1}, respectively. The observed heats were the same in both Tris and phosphate buffers, indicating no release or uptake of protons during the reaction. Since the equilibrium constants for these reactions are similar the differences in ΔH are for the most part entropy compensated. The unitary entropy changes for the reactions in aqueous solution at 25° are $\Delta S_u = -14.5$ and $\Delta S_u = -27.0$ cal deg^{-1} mole^{-1} for undialyzed horse and sperm-whale myoglobins, respectively. The differences in ΔH between dialyzed and undialyzed samples is believed to be due to the binding of an unidentified small molecule, as suggested by Lumry and coworkers (M. H. Keyes, Ph.D. Thesis, University of Minnesota, 1968).

Myoglobin, the oxygen binding heme protein found almost universally distributed in muscle tissue, has been under intensive study for the past 40 years. In addition to revealing the function of this protein as a "storehouse" of oxygen, these studies have also done much to uncover the relationships between the structure and function of myoglobin. However, thermodynamic investigations of the interaction of myoglobin with O_2 and CO have generally not gone beyond the measurement of equilibrium constants. Equilibrium measurements have been made on the Mb–O_2[1] reaction for many species and in a few cases also for the Mb–CO reaction (see review articles by Rossi-Fanelli *et al.*, 1964, and Antonini, 1965). In the cases where equilibrium measurements were made as a function of temperature, values of ΔH are available by the van't Hoff method (Theorell, 1934; Rossi-Fanelli and Antonini, 1958; Rossi-Fanelli *et al.*, 1959; Keyes, 1968). However, these data are sketchy (particularly for MbCO) and there is some disagreement as to the correct values. We therefore decided to measure the heat of the Mb–CO reaction by direct calorimetry in order to complete the thermodynamic picture. This study also afforded us the opportunity of testing a unique gas–liquid reaction calorimeter (described below) which is now being used to study similar reactions with hemoglobin.

The Mb–CO reaction was chosen rather than the physiological Mb–O_2 reaction because earlier calorimetric experiments showed that consistent results were not obtainable with O_2. Unlike hemoglobin, Mb is subject to fairly rapid oxidation, particularly at low O_2 concentrations. Horse and sperm-whale myoglobins were chosen because of their ready availability and the existence of a large body of data on their physical and chemical properties, including amino acid sequence and X-ray diffraction studies.

Experimental Section

Materials. Horse and sperm-whale myoglobin were obtained as salt-free, crystalline powders from Seravac, Ltd., and were stored dry at −6°. The absorption spectra of these materials showed them to be primarily in the met or oxidized state. Preparation for use in calorimetric runs was carried out in ice-cold solutions as follows. Dry myoglobin (300–600 mg) was dissolved in 20 ml of the chosen buffer. This solution was centrifuged at 10,000*g* for 10 min to remove small amounts of insoluble material. In experiments where dialyzed protein was used, the Mb was dialyzed against several 2-l. changes of buffer for a total of 18–24 hr. The clear solution was then transferred to a graduated vessel having a 10-mm spectrophotometric cell with a 9-mm quartz spacer at the bottom and a three-way stopcock at the top. Dissolved oxygen was removed from the solution by bubbling with pure nitrogen for 20 min. Reduction of the Mb^+ was then carried out by the method of Rossi-Fanelli *et al.* (1957) using NADH, Methylene Blue, and cytochrome *c* reductase. An alternate method for reduction was found by simply adding ascorbic acid to a concentration of 0.01 M to the Mb^+ solution and bubbling with

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[1] Abbreviations used are: Mb, reduced or ferromyoglobin; Mb^+, oxidized or metmyoglobin; MbO_2, oxymyoglobin; MbCO, carbon monoxymyoglobin.