The Interaction of *N*-Acetylglucosamine and an Affinity-Label Analogue with α-Lactalbumin and Lactose Synthetase†

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ABSTRACT: We have attempted to detect binding of *N*-acetylglucosamine (NAG) to α-lactalbumin, the B protein of lactose synthetase, under conditions in which binding of NAG to lysozyme, a protein to which α-lactalbumin has a significant sequence homology, is observed. Using ^{1}H nuclear magnetic resonance spectroscopy, uv difference spectroscopy, competition of NAG with *N*-methylnicotinamide chloride, and fluorescence spectroscopy, no binding was detected. The synthesis of a NAG analogue, *N*-diazoacetylglucosamine (diazoNAG), was carried out, and the molecule was demonstrated to be an active galactose acceptor in the lactose synthetase reaction. Use of this molecule in photochemical labeling experiments resulted in a large amount of nonspecific labeling of α-lactalbumin, lactose synthetase A protein, ribonuclease, and lysozyme, but competition experiments in the presence of an excess of NAG revealed some specific labeling in the case of A protein and lysozyme, but not with α-lactalbumin or a ribonuclease control. Thus, it is highly questionable that a NAG binding site is retained in α-lactalbumin; furthermore, it appears that the galactosyl acceptor makes significant contacts with the A protein rather than α-lactalbumin in the lactose synthetase complex.

Lactose synthetase (UDP-galactose:D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the last step in lactose biosynthesis:

$$\text{UDP-galactose} + \text{glucose} \xrightarrow{Mn^{2+}} \text{lactose} + \text{UDP} \quad (1)$$

The enzyme has been resolved into two components, both of which are necessary for the observed lactose synthetase activity (Ebner et al., 1966; Brodbeck et al., 1967). One component, the A protein, has been subsequently (Brew et al., 1968; Hill et al., 1969) shown to be a galactosyltransferase similar to, and probably identical with, that present in many tissues, the role of which is evidently to transfer galactose from UDP-galactose to the growing polysaccharide side chains of glycoproteins. In particular, one in vitro reaction of the A protein, particularly useful in its analysis, is that of eq 2.

$$\text{UDP-galactose} + \textit{N}\text{-acetylglucosamine (NAG)}^1 \xrightarrow{Mn^{2+}} \textit{N}\text{-acetyllactosamine (NAL)} + \text{UDP} \quad (2)$$

The other component of lactose synthetase, shortly identified as α-lactalbumin (Ebner et al., 1966), a major whey protein of milk, has been shown (Brew et al., 1968) to have an effect which was summarized as that of a "specifier protein": in the presence of α-lactalbumin, the rate of the lactose synthetase is dramatically increased. It was subsequently demonstrated (Klee and Klee, 1970; Morrison and Ebner, 1971; Khatra et al., 1974) that the effect of α-lactalbumin on reactions 1 and 2 is qualitatively the same: α-lactalbumin lowers the K_m of the carbohydrate acceptors in both reactions, while leaving their V_{max} unaffected, so that the difference in the effect of α-lactalbumin on glucose and NAG acceptor specificities is a quantitative one. One conceivable (but certainly not unique) explanation for these observations is that α-lactalbumin could be providing a partial binding site for the saccharide acceptor.

The sequence of bovine α-lactalbumin (Brew et al., 1970) generated a substantial degree of interest when it was observed that this protein (mol wt 14400, 123 amino acid residues) and the lysozymes (mol wt 14300, 129 residues) of the hen egg type possessed great similarities in primary structure. These similarities have led to proposed three-dimensional models of α-lactalbumin (Browne, et al., 1969; Warme et al., 1974). The similarity of these two proteins takes on greater appeal in view of the effect of α-lactalbumin on reaction 2, to lower the K_m of NAG, together with the fact that NAG is known to bind to, and be a competitive inhibitor of, lysozyme. Furthermore, both lysozyme and α-lactalbumin participate in reactions that involve β (1 → 4) glycosidic linkages. A direct catalytic role for α-lactalbumin is probably unlikely, in view of the facts that (1) α-lactalbumin does not affect the V_{max} of lactose synthetase; (2) neither lysozyme nor α-lactalbumin possesses each other's activity;[2] and (3) there is no lactose synthetase activity when α-lactalbumin and A protein are separated by a dialysis membrane (Brew et al., 1968). The attractiveness of a role for α-lactalbumin in the direct binding of NAG (or glucose), based on the lysozyme analogy, suggested that a

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[1] Abbreviations used are: NAG, 2-acetamido-2-deoxy-D-glucopyranose, or *N*-acetylglucosamine; NAL, *N*-acetyllactosamine; diazoNAG, 2-diazoacetamido-2-deoxy-D-glucopyranose, or *N*-diazoacetylglucosamine; NMN, *N*-methylnicotinamide.

[2] A recent paper appeared (Hopper and McKenzie, 1974) in which it is reported that milk from the spiny anteater contains a protein possessing both lysozyme and α-lactalbumin activities.

search for direct binding of NAG to α-lactalbumin might prove to be profitable. We anticipated that conventional equilibrium dialysis techniques would not be useful, since, in this method, the substrate dissociation constant and protein concentration must be of the same order of magnitude; the lysozyme analogy suggested that any NAG–α-lactalbumin interaction would probably be weak, so that we turned to methods especially useful for detecting weak bindings. By use of an affinity label designed as a NAG analogue, we also decided to investigate the NAG–α-lactalbumin interaction within the lactose synthetase complex. In this paper, we report that there appears to be no binding of NAG to α-lactalbumin. In addition, the results of affinity labeling work show that the most likely site of interaction of NAG with lactose synthetase is with the A protein.

Materials and Methods

Binding Studies. Bovine α-lactalbumin was prepared from skim milk obtained from the Cornell Dairy by either of two methods (Aschaffenburg, 1968; Castellino and Hill, 1970) and showed one major and one very minor band by polyacrylamide gel electrophoresis. Three times crystallized hen egg lysozyme was obtained from General Biochemicals, and NAG from Schwarz/Mann. *N*-Methylnicotinamide (NMN) iodide was prepared in 81% yield from nicotinamide (Sigma) (Pullman et al., 1954). The NMN iodide was converted to the chloride (Karrer et al., 1936) in 79% yield after recrystallization from 95% ethanol ($\epsilon_{H_2O}^{350\ nm}$ 0.01 M^{-1} cm^{-1}).

Nuclear magnetic resonance (NMR) proton spectra were run on a Varian Associates A60A instrument using acetone (0.5% v/v) as internal standard, whereas, at 90 MHz, a Bruker HX-90 instrument, operating in the FT (Fourier transform) pulse mode, was used, with acetone at 0.04% (v/v). All studies were performed at ambient probe temperature (ca. 35°).

Difference ultraviolet (uv) spectra (Yankeelov, 1963) were determined on a Cary 1605 spectrophotometer. Fluorescence measurements were carried out on a Perkin-Elmer MPF-3 fluorescence spectrometer; scans for fluorescence emission covered the range 300–400 nm, and excitation was at 280 nm. Appropriate controls were run for quenching by NAG using tryptophan solutions.

Affinity Label Studies. p-Nitrophenyl diazoacetate was prepared by a known method (Shefer et al., 1966).

N-Diazoacetylglucosamine (diazoNAG, **1**) was prepared by allowing 3.6 g of *p*-nitrophenyl diazoacetate and 2.5 g of glucosamine hydrochloride to react in a solution of 2.35 g of triethylamine and 35 ml of dimethyl sulfoxide (Me_2SO), in a stirred, stoppered round-bottomed flask for 5.75 hr in the dark. After this time, NMR of the reaction mixture indicated that reaction was complete. The dark brown reaction mixture was poured into 700 ml of CH_2Cl_2 and allowed to stand at room temperature for 0.5 hr. The dark brown solid that precipitated was removed by filtration and quickly washed on the filter with 100 ml of CH_2Cl_2 and 100 ml of diethyl ether. The solid was dissolved in 200 ml of methanol and placed on a 4 × 23 cm column of Norit A/Celite (1:1 w/w) and eluted with methanol. The fractions of the first yellow peak to elute were pooled and concentrated to dryness on the rotary evaporator. A small amount of triethylamine contaminating this material was removed by washing it with 60 ml of acetone, giving the product in 35% yield; the material darkens at 150°, and decomposes at ca. 200°: uv, λ_{max} (0.1 *M* phosphate buffer, pH 7) 252 nm (ε 5000); ^1H NMR (60 MHz, Me_2SO-d_6) δ 2.8–5.0 (series of multiplets, 11 H), 5.4 (large singlet (α anomer)), 5.3 (small singlet (β anomer), total 1 H), 6.4 (d, 1 H), and 7.4 (broad d, 1 H), the α:β anomeric ratio (Inch, 1969) was 3:1; ir (KBr) 3300, 2100, 1620, 1580 cm^{-1}, all strong. Paper chromatography (ascending, on Whatman No. 1 using 1-butanol–pyridine–water, 6:4:3 v/v, and visualized with Morgan–Elson reagent (Cramer, 1954)) showed one major spot at R_f 0.61, and a very faint spot at R_f 0.15. The ^{13}C NMR spectrum was identical with that of NAG, except that the diazoacetyl side chain resonances were shifted relative to NAG by amounts indicated to be appropriate by a comparison of ^{13}C NMR spectra of several acetate and diazoacetate esters and amides. For a detailed spectral analysis, see the discussion by Burkhardt (1975).

As part of the structural arguments for diazoNAG, this compound was converted into NAG with HI (Wolfrom and Brown, 1943; Fieser and Fieser, 1967). Into a small test tube was placed 17 mg of diazoNAG; 4 ml of 47% HI was diluted to 30 ml with methanol, and 0.5 ml of this solution was added to the test tube, whereupon immediate bubble (N_2) formation was noted. The solution was stirred at room temperature for 1 hr and added to 100 ml of ether. After standing at room temperature for 0.5 hr, the white solid was filtered and washed with 30 ml of ether. Assuming it to be NAG, the yield was 87%. The ir spectrum of this material was identical with that of NAG subjected to the same conditions.

Synthesis of [6-^{3}H]-*N*-diazoacetylglucosamine was carried out by an adaption of the above procedure for the unlabeled material (Burkhardt, 1975), using [6-^{3}H]-D-glucosamine hydrochloride (specific activity 250 mCi, 10.13 Ci/mmol) as starting material. This material was obtained in ≥91% radiochemical purity.

The A protein of lactose synthetase was purified by an established procedure (Trayer and Hill, 1971) from bovine skim milk. Dodecyl sulfate polyacrylamide gel electrophoresis confirmed that the known active bands comprised about 65% of the total protein. The usual bands at mol wt ca. 55000 and 45000 were observed, with the putatively inactive band at ca. 25000 mol wt (Magee et al., 1974) accounting for the balance of the protein.

The photolysis procedure for the affinity label experiments was similar to that described by others (Singh et al., 1962). The solutions to be photolyzed were placed in a 13 × 100 mm culture tube, which was placed in a 100-ml jacketed reaction vessel thermostated at 26 ± 1° filled with 40% (w/v) aqueous $CuSO_4$ solution. Photolysis was accomplished by irradiating the vessel with a GE 275W sunlamp positioned 4 cm from the wall of the jacketed vessel; the light thus passed through a minimum of 8 mm of Pyrex and 1 cm of $CuSO_4$ before entering the protein solution. The A protein was assayed immediately before and after photolysis, which was carried out for 6.5 hr, after which time measurement of the diazoNAG uv absorption showed that photolysis was complete. The compositions of the solutions used in individual experiments is given in the Results section.

After completion of the photolysis, the solution was transferred to a cellulose casing dialysis bag and dialyzed three times against 500 ml of the buffer used in the photolysis to remove any non-protein-bound radioactive materials. The dialyzed solution was then placed on a 1.5 × 86 cm Bio-Gel P-30 column and eluted with the same buffer at a flow rate of 0.5 ml/min (2.4-ml fractions). Selected fractions were monitored for protein by absorbance at 280 nm

and for radioactivity. The fractions containing A protein and those containing α-lactalbumin were pooled separately and assayed for protein (in the case of the latter) using $\epsilon_{280\,nm}^{1\%}$ 20.1 (Kronman and Andreotti, 1964).

Results

Direct Binding Experiments. 1. NMR Measurements. It has been demonstrated (Dahlquist and Raftery, 1968) that one may deduce NAG–lysozyme binding constants, as well as information about the magnetic environment of the bound saccharide, by observing both the chemical shift and transverse relaxation time of the acetamido methyl group of NAG in the presence of lysozyme, and as a function of NAG concentration. After finding that we were able to reproduce the lysozyme experiments at 60 MHz, we turned to similar experiments with NAG and α-lactalbumin. The results of these experiments, designed to probe the effect of α-lactalbumin on the chemical shift of the acetamido protons of NAG, were uniformly negative; the chemical shift of the acetyl methyl group of NAG was found to be 10.4 ± 0.1 Hz upfield from internal acetone both in the presence and absence of 50 mg/ml of α-lactalbumin (pH 6.98, 0.1 *M* phosphate). Use of the lower substrate concentrations necessary to provide larger fractions bound to the protein was possible at 90 MHz using the Bruker HX-90 NMR spectrometer operated in the Fourier transform mode. In this study, a solution of 0.0012 *M* NAG was prepared in a buffer formulated by mixing equal volumes of a 0.1 *M* sodium phosphate buffer and D_2O (the latter required as a lock signal). The pH meter reading of the final buffer was 6.9 (uncorrected; a pD correction (Glascoe and Long, 1960) would be on the order of 0.15 unit higher). The spectra, obtained after 1800 pulses, gave the same result, that no chemical shift was observed for NAG relative for acetone when 25 mg/ml of α-lactalbumin was added. Finally, in view of a recent report that the pH optimum of galactosyltransferase is shifted from ca. 7 to ca. 10 by the presence of α-lactalbumin (Osborne and Steiner, 1974) we repeated the 60-MHz experiments at pH 10 (0.1 *M* sodium bicarbonate buffer) with, however, the same negative result.

Because binding might occur with no attendant chemical shift, we considered the effect of α-lactalbumin on the transverse relaxation time (T_2) of the acetamido protons of NAG at 60 MHz. The results of these experiments were that the values of T_2, relative to those of the internal acetone standard under similar conditions, are unaffected.

2. Ultraviolet Difference Spectroscopy Measurements. The substantial uv difference spectrum of lysozyme induced by the presence of NAG is well known (Dahlquist, et al., 1966). Similar experiments (1.0 mg/ml of α-lactalbumin, 0.1 *M* NAG, and 0.1 *M* Tris-HCl (pH 7.0)) showed that no difference spectrum is observed for α-lactalbumin in the presence of NAG. As in the NMR experiments, such a negative result may only mean that binding is not perturbing an enzyme chromophore. We desired to have an indicator known to bind to α-lactalbumin, which we might displace, or perturb, with NAG. The observation that NMN chloride interacts weakly ($K_d \approx 3$–4 *M*) with both lysozyme and α-lactalbumin to produce an intense charge-transfer band ($\epsilon_{350\,nm}$ 1135) suggested that NMN chloride would be useful (Robbins and Holmes, 1972; Bradshaw and Deranleau, 1970). Furthermore, the NMN chloride is known to be displaced from lysozyme by NAG. Thus, we reproduced the binding of NMN chloride to α-lactalbumin; the addition of up to 0.3 *M* NAG had no effect on the difference spectrum and the derived double-reciprocal plot resulting from NMN chloride binding.

Table I: Affinity Labeling Experiments with DiazoNAG.[a,b]

Entry	Photolysis Solutions: Components	Concn (mg/ml)	Buffer	Labeling[a]
1	A protein	0.840	0.05 *M* sodium cacodylate, pH 7.5, 5 m*M* $MnCl_2$, 1 m*M* β-mercaptoethanol, 0.02% NaN_3	0.210
	α-Lactalbumin	26.3		
	DiazoNAG	7.32		
2	α-Lactalbumin	26.3	Same as entry 1	0.232
	DiazoNAG	6.42		
3	A protein	0.840	Same as entry 1	0.238
	α-Lactalbumin	26.0		
	DiazoNAG	10.0		
	NAG	223.0		
4	Ribonuclease A	21.0	Same as entry 1	0.285
	DiazoNAG	10.4		
5	Lysozyme	26.5	Same as entry 1	0.286
	DiazoNAG	4.83		
6	Lysozyme	27.2	Same as entry 1	0.206
	DiazoNAG	5.03		
	NAG	228.0		
7	A protein	0.444	Same as entry 1	0.00048
	α-Lactalbumin	26.3		
	DiazoNAG	0.038		

[a] Labeling = moles of diazoNAG covalently bound per mole of protein (other than A protein). [b] Specific labeling of A protein *was* observed in the experiments of entries 1 and 3; see text.

3. Fluorescence Measurements. The interaction of NAG and α-lactalbumin was monitored by fluorimetry, paralleling previous methods used for lysozyme (Lehrer and Fasman, 1966) and across a range of pH values from 7.5 to 9.0 (0.1 *M* Tris-HCl buffer). No shift or intensity variation in the fluorescence emission was found.

Affinity Label Experiments. The interaction of NAG and the proteins within the lactose synthetase complex was examined using an affinity label synthesized for this purpose, [6-^{3}H]-*N*-diazoacetyl-D-glucosamine, **1** (diazoNAG, see Materials and Methods).

In order that diazoNAG qualify as a suitable label, it had to be stable under the conditions to be used for labeling, and it had to bind at least to the A protein of lactose synthetase. That the first requirement was met was established by some preliminary kinetic experiments in which disappearance of diazoNAG uv absorption (250 nm) was monitored as a function of time. At pH 7, the half-life of the material was ca. 24 hr, but at lower pH values it decreased, as expected for the acid-catalyzed solvolytic removal of the diazo group (Bell, 1973). That the second requirement was met was ascertained by the finding that galactosyltransferase activity of the A protein was retained to the extent of 67% when diazoNAG was substituted for NAG as an acceptor under standard NAL synthetase conditions.

Table I summarizes the results of the affinity labeling experiments. Entry 1 shows that α-lactalbumin is indeed labeled by diazoNAG; entry 2, however (A protein absent), shows that either the binding state for diazoNAG is entirely on the α-lactalbumin, a result at variance with all the binding studies detailed above, or that the labeling is entirely nonspecific in nature. That the latter alternative is probably correct is shown by entry 3, in which the system is flooded with excess NAG; the NAG did not protect the α-lactalbumin from labeling. In the experiment described by entry 4,

ribonuclease A, a protein which does not bind NAG, was labeled at least to the extent of that observed for α-lactalbumin. Lysozyme, however, which is known to bind NAG weakly, is also labeled by diazoNAG, but is evidently partially protected from labeling by NAG present in large excess. In view of the known propensity of photolytically generated carbenes to undergo a rearrangement to ketenes (Knowles, 1972) and the rapid reaction of these species with nucleophiles, especially amines (Lacey, 1960), it seems reasonable that the nonspecific labeling of proteins through ketene intermediates may occur primarily at the surface amino groups. Ketene itself has, in fact, been used to acetylate insulin (Stern and White, 1938); the amine groups on the protein react rapidly at pH 5.7. Addition of excess amine in order to foil this type of side reaction is not possible because of the facile formation of glycosylamines.

In a final attempt (entry 7), a labeling experiment was conducted at very low diazoNAG concentrations. The nonspecific labeling should decrease in proportion to the diazoNAG concentration, whereas the fraction of the label bound specifically should increase. From the relative concentrations of water and protein, the labeling that should be observed if solely random labeling is occurring should be about 0.001; the observed labeling is close to this value.

In the experiments of entries 1 and 3 the labeling of the A protein of lactose synthetase was estimated at 0.45 in the absence of NAG, and 0.30 in its presence. Although the A protein is nonspecifically labeled in large amounts, *there is evidently significant protection by NAG.* It should be noted that the A protein at the end of the photolysis period retained ca. 67% of its initial activity; 50% of the lost activity (15% of the total) might be accounted for by the specific labeling.

Binding Studies. All the binding studies carried out in the previous section employed methods which have been successful in detecting the binding of NAG to lysozyme, and the results of these experiments were uniformly negative. Because each of the binding studies require a specific response of the protein, the results do not of course prove the absence of binding. However, the results do suggest that if α-lac and lysozyme share a common affinity for NAG, the binding sites on the two proteins are considerably different.

Affinity Label Studies. At the time of inception of this work, existing kinetic studies (Morrison and Ebner, 1971) as well as sedimentation equilibrium work (Klee and Klee, 1972) strongly suggested that there exists a kinetically important A protein–Mn^{2+}–NAG–α-lactalbumin complex, and that there would be therefore an excellent chance of labeling α-lactalbumin within the lactose synthetase complex using a diazo analogue of NAG. It was anticipated that, if such labeling occurred, it would be catalyzed by the A protein in the sense that more than one molecule of α-lactalbumin per molecule of A protein could be labeled, since the A protein–Mn^{2+}–diazoNAG–α-lactalbumin complex would be expected to dissociate rapidly. The failure to label α-lactalbumin specifically suggests that at least the diazoacetyl side chain of the analogue is not in intimate contact with α-lactalbumin within this complex. That this result was not due to a failure of the analogue to bind is suggested by (a) the high activity of diazoNAG as a replacement for NAG in the A protein NAL synthetase assay; (b) the recent finding (Khatra et al., 1974) that *N*-propionylglucosamine may substitute for NAG in the A protein assay as well; and (c) the observed labeling of the A protein, some of which was apparently specific. The latter observation further implies that *the A protein makes the important contacts with the acetyl amino sugar* in the lactose synthetase complex. It is noteworthy that a recent series of extensive kinetic studies (Khatra et al., 1974) has suggested that complexes containing α-lactalbumin, Mn^{2+}, A protein, and NAG which have been observed are nonproductive, and that productive binding of α-lactalbumin requires the presence of UDP-galactose. On the basis of the experiments reported here, and realizing their inherent limitations, we believe that, although α-lactalbumin conformation may greatly resemble lysozyme in its gross features, the NAG binding site will have been essentially obliterated. It is entirely possible, however, that α-lactalbumin may positively interact with UDP-galactose within the lactose synthetase complex; that it acts to cause a conformational change in the A protein; or that a conformational state produced by the presence of UDP-galactose is important for catalytic activity, and permits the binding of α-lactalbumin to the A protein. The latter idea receives some support in recent modification studies (Magee and Ebner, 1974). Experiments are underway to evaluate these alternatives.

In the first experiments with photoaffinity labels (Singh et al., 1962; Shefer et al., 1966; Stefanovsky and Westheimer 1973; Vaughan and Westheimer, 1969) the labels were first covalently bound to the enzyme prior to photolysis. When the same group attempted the photoaffinity labeling of yeast alcohol dehydrogenase (Browne et al., 1971) with a diacetoxymethyl analogue of nicotinamide adenine dinucleotide, a substrate that does not form such a covalent bond, substantial amounts of nonspecific labeling evidently occurred. These workers concluded that a tightly bound or covalently bound affinity label is generally desirable in these kinds of experiments, and that otherwise great difficulties are to be expected with nonspecific labeling. Recent kinetic studies (Khatra et al., 1974) place the K_M of NAG toward lactose synthetase in the millimolar range, and this is in the range considered weak by Browne et al. Nevertheless, a small amount of specific labeling evidently occurred with lysozyme and diazoNAG, and with A protein and diazoNAG, and none occurred with ribonuclease and diazoNAG, a protein which presumably does not bind NAG; these points add strength to our arguments that NAG does not directly interact with α-lactalbumin. The K_m of UDP-galactose (Khatra et al., 1974) is in the micromolar range, so that experiments to probe the lactose synthetase active site(s) with UDP-galactose analogues, now in progress, appear to offer considerably more promise.

References

Aschaffenburg, R. (1968), *J. Dairy Sci. 51,* 1295.

Babad, H., and Hassid, W. Z. (1966), *J. Biol. Chem. 241,* 2672.

Barman, T. E. (1972), *Biochim. Biophys. Acta 258,* 297.

Bell, R. P. (1973), The Proton in Chemistry, 2nd ed, Ithaca, N.Y., Cornell University Press, p 171.

Bradshaw, R. A., and Deranleau, D. A. (1970), *Biochemistry 9,* 3310.

Brew, K., Castellino, F. J., Vanaman, T. C., and Hill, R. L. (1970), *J. Biol. Chem. 245,* 4570, and references therein.

Brew, K., Vanaman, T. C., and Hill, R. L. (1968), *Proc. Natl. Acad. Sci. U.S.A. 59,* 491.

Brodbeck, U., Denton, V. L., Tanahashi, N., and Ebner, K. E. (1967), *J. Biol. Chem. 242,* 1391.

Browne, D. T., Hixson, S. S., and Westheimer, F. H.

(1971), *J. Biol. Chem. 246,* 4477.
Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C., and Hill, R. L. (1969), *J. Mol. Biol. 42,* 65.
Burkhardt, A. E. (1975), Ph.D. Dissertation, Cornell University.
Castellino, F. J., and Hill, R. L. (1969), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28,* 405.
Castellino, F. J., and Hill, R. L. (1970), *J. Biol. Chem. 245,* 417.
Chaimovich, H., Vaughan, R., and Westheimer, F. H. (1968), *J. Am. Chem. Soc. 90,* 4088.
Cramer, F. (1954), Paper Chromatography, London, Macmillan, p 63.
Dahlquist, F. W., Jao, L., and Raftery, M. A. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 26.
Dahlquist, F. W., and Raftery, A. (1968), *Biochemistry 7,* 3269.
Ebner, K. E. (1973), *Enzymes, 3rd Ed. 9,* 363.
Ebner, K. E., Denton, W. L., and Brodbeck, U. (1966), *Biochem. Biophys. Res. Commun. 24,* 232.
Fieser, L. F., and Fieser, M. (1967), Reagents for Organic Synthesis, Vol. 1, New York, N.Y., Wiley, p 298.
Glascoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64,* 188.
Hill, R. L., Brew, K., Vanaman, T. C., Trayer, I. P., and Mattock, P. (1969), *Brookhaven Symp. Biol. 21,* 139.
Hopper, K. E., and McKenzie, H. A. (1974), *Mol. Cell. Biol. 3,* 93.
Inch, T. D. (1969), *Annu. Rev. NMR Spectrosc. 2,* 44.
Karrer, P., Schwarzenbach, G., Berg, F., and Solnissen, U. (1936), *Helv. Chim. Acta 19,* 811.
Khatra, B. S., Herries, D. G., and Brew, K. (1974), *Eur. J. Biochem. 44,* 537.
Klee, W. A., and Klee, C. B. (1970), *Biochem. Biophys. Res. Commun. 39,* 833.
Klee, W. A., and Klee, C. B. (1972), *J. Biol. Chem. 247,* 2336.
Knowles, J. R. (1972), *Acc. Chem. Res. 5,* 155.
Kronman, M. J., and Andreotti, R. E. (1964), *Biochemistry 3,* 1145.
Lacey, R. N. (1960), in Advances in Organic Chemistry, Methods and Results, Vol. 2, Raphael, R. A., Taylor, E. C., and Wynberg, H., Ed., New York, N.Y., Interscience, p 218.
Lehrer, S. S., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun. 23,* 133.
Magee, S. C., and Ebner, K. E. (1974), *J. Biol. Chem. 249,* 6992.
Magee, S. C., Mawal, R., and Ebner, K. E. (1974), *Biochemistry 13,* 99.
Morrison, J. F., and Ebner, K. E. (1971), *J. Biol. Chem. 246,* 3977, 3982, 3985.
Osborne, J. C., and Steiner, R. F. (1974), *Arch. Biochem. Biophys. 165,* 615.
Pullman, M. E., San Pietro, A., and Colwick, P. (1954), *J. Biol. Chem. 206,* 129.
Robbins, F. M., and Holmes, L. G. (1972), *J. Biol. Chem. 247,* 3062.
Shefer, J., Baronowsky, P., Laursen, R., Finn, F., and Westheimer, F. H. (1966), *J. Biol. Chem. 241,* 421.
Singh, A., Thornton, E. R., and Westheimer, F. H. (1962), *J. Biol. Chem. 237,* PC3006.
Stanek, J., Cerny, M., Kocourek, J., and Pacak, J. (1963), The Monosaccharides, Prague, Academic Press and the Czechoslovak Academy of Sciences Publishing House, p 449.
Stefanovsky, T., and Westheimer, F. H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 132.
Stern, K. G., and White, A. (1938), *J. Biol. Chem. 122,* 371.
Trayer, I. P., and Hill, R. L. (1971), *J. Biol. Chem. 246,* 6666.
Vaughan, R. J., and Westheimer, F. H. (1969), *J. Am. Chem. Soc. 91,* 217.
Warme, P. K., Momany, F. A., Rumball, S. V., Tuttle, R. W., and Scheraga, H. A. (1974), *Biochemistry 13,* 768.
Wolfrom, M. L., and Brown, R. L. (1943), *J. Am. Chem. Soc. 65,* 1516.
Yankeelov, J. A., Jr. (1963), *Anal. Biochem. 6,* 287.