populations from both groups. Significant differences, however, were observed in the concentration of insulin required to inhibit 50 percent of maximum binding in lymphocytes from obese and normal weight people. At present, we feel that studies in which circulating lymphocytes are used to examine insulin binding in resistant states should be viewed with some caution. Unlike the adipocyte, circulating lymphocytes have extremely low levels of specific insulin binding and, to date, no biological response to insulin has been observed (*19*). Furthermore, preparations of the circulating lymphocytes used in these insulin binding studies were significantly contaminated with other cell types (*18*), some of which bind insulin avidly (*19*). The second report, which was presented at a symposium in 1971 (*20*), alludes to studies of insulin binding to human fat cells from obese and lean individuals. No affinity studies were performed and there is no information on the quantity of insulin bound per cell. Furthermore, saturability, a requisite of specific receptor binding, was not present.

It has been suggested that elevated circulating insulin may contribute to the decrease in insulin receptors seen in some insulin-resistant states (*21*). Studies of cultured human lymphocytes (*19, 21*) which, unlike peripheral circulating lymphocytes (*19*), possess a high density of insulin receptors have shown that the incubation of lymphocytes with very high concentrations of insulin ($> 10^{-8}M$) for five or more hours at 37°C leads to a decrease in insulin receptors (*21*). The concept that elevated concentrations of insulin lead to a decrease in receptors does not appear to apply to human obesity. Despite elevated concentrations of insulin (Table 1), there is no decrease in insulin binding to either human adipocytes as shown in the present study (Fig. 1) or to circulating lymphocytes (*18*).

An impairment of insulin action in large adipocytes from obese human subjects has been demonstrated (*3–6*). Although the present study indicates that this insulin resistance follows the insulin-cell association, it does not provide direct information concerning the cellular alteration (or alterations) responsible for hormonal insensitivity. One possibility is that a "dilution" of insulin receptors over the surface area of large cells may hinder the transmission of the signal arising from the interaction between insulin and the insulin receptor (*16*). This does not appear to be the case in at least one animal model in which large adipocytes have an efficient D-glucose transport system that is responsive to insulin despite reduced insulin-stimulated glucose oxidation (*22*). Numerous studies in which both animal (*23*) and human (*6, 24*) adipocytes were used show that intracellular carbohydrate and lipid metabolism is altered in large fat cells as compared to small ones. It is possible that these metabolic alterations might render large fat cells less sensitive to some of the actions of insulin which would in turn appear as insulin resistance.

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References and Notes

1. J. D. Bagdade, E. L. Bierman, D. Porte, Jr., *J. Clin. Invest.* **46**, 1549 (1967).
2. E. A. H. Sims, R. F. Goldman, C. M. Gluck, E. S. Horton, P. C. Kelleher, D. W. Rowe, *Trans. Assoc. Am. Physicians Phila.* **81**, 153 (1968); P. Björntorp, P. Berchtold, G. Tibblin, *Diabetes* **20**, 65 (1971); J. Stern, B. Batchelor, N. Hollander, C. Cohn, J. Hirsch, *Lancet* **1972-II**, 948 (1972).
3. D. Rabinowitz and K. L. Zierler, *J. Clin. Invest.* **41**, 2173 (1962).
4. L. B. Salans, J. L. Knittle, J. Hirsch, *ibid.* **47**, 153 (1968).
5. L. B. Salans, G. A. Bray, S. W. Cushman, E. Danforth, Jr., J. A. Glennon, E. S. Horton, E. A. H. Sims, *ibid.* **53**, 848 (1974).
6. U. Smith, *J. Lipid Res.* **12**, 65 (1971).
7. P. Björntorp, *Acta Med. Scand.* **179**, 229 (1966); M. B. Davidson, *Diabetes* **21**, 6 (1972).
8. M. E. Krahl, *Perspect. Biol. Med.* **1**, 69 (1957); R. Levine, *Fed. Proc.* **25**, 1071 (1965); O. Hechter, *Mechanism of Hormone Action*, P. Karlson, Ed. (Academic Press, New York, 1965); P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **63**, 450 (1969); P. Freychet, J. Roth, D. M. Neville, Jr., *ibid.* **68**, 1833 (1971).
9. P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1264 (1971).
10. J. M. Olefsky, P. Jen, G. M. Reaven, *Diabetes* **23**, 565 (1974).
11. G. A. Bray, *Ann. Intern. Med.* **73**, 565 (1970).
12. S. Gammeltoft and J. Gliemann, *Biochim. Biophys. Acta* **320**, 16 (1973).
13. C. R. Kahn, D. M. Neville, Jr., J. Roth, *J. Biol. Chem.* **248**, 244 (1973).
14. C. R. Kahn, I. D. Goldfine, D. M. Neville, Jr., J. Roth, R. W. Bates, M. M. Garrison, *Endocrinology* **93** (Suppl.), A-168 (1973); J. M Olefsky, J. Johnson, F. Liu, P. Jen, G. Reaven, *ibid.* **94** (Suppl.), A-282 (1974).
15. I. Goldfine, C. R. Kahn, D. M. Neville, Jr., J. Roth, M. M. Garrison, R. W. Bates, *Biochem. Biophys. Res. Commun.* **53**, 852 (1973).
16. J. N. Livingston, P. Cuatrecasas, D. H. Lockwood, *Science* **177**, 626 (1972).
17. G. V. Bennett and P. Cuatrecasas, *ibid.* **176**, 805 (1972).
18. J. A. Archer, P. Gorden, J. R. Gavin, III, M. A. Lesniak, J. Roth, *J. Clin. Endocrinol. Metab.* **36**, 627 (1973).
19. U. Krug, F. Krug, P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2604 (1972).
20. G. V. Marinetti, L. Schlatz, K. Reilly, in *Insulin Action*, I. Fritz, Ed. (Academic Press, New York, 1972), pp. 224 and 253.
21. J. R. Gavin, III, J. Roth, D. M. Neville, Jr., P. DeMeyts, D. Buell, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 84 (1974).
22. J. N. Livingston and D. H. Lockwood, *Biochem. Biophys. Res. Commun.* **61**, 989 (1974).
23. A. D. Hartman, A. I. Cohen, C. J. Richare, T. Hsu, *J. Lipid Res.* **12**, 498 (1971); M. DiGirolamo and S. Mendlinger, *Diabetes* **21**, 1151 (1972); R. S. Bernstein and D. M. Kipnis, *ibid.* **22**, 913 (1973).
24. P. Björntorp and M. Karlsson, *Eur. J. Clin. Invest.* **1**, 112 (1970); R. B. Goldrick and G. M. McLoughlin, *J. Clin. Invest.* **49**, 1213 (1970); P. Björntorp and L. Sjöström, *Eur. J. Clin. Invest.* **2**, 78 (1972); J. L. Knittle and F. Ginsberg-Fellner, *Diabetes* **21**, 754 (1972).
25. R. B. Goldrick, *Am. J. Physiol.* **212**, 777 (1967).
26. J. Gliemann, K. Østerlind, J. Vinten, S. Gammeltoft, *Biochim. Biophys. Acta* **286**, 1 (1972).
27. K. Burton, *Biochem. J.* **62**, 315 (1956).
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Nuclear Magnetic Resonance of Rotational Mobility of Mouse Hemoglobin Labeled with [2-^{13}C]Histidine

Abstract. *Carbon-13 nuclear magnetic resonance studies were made on mouse hemoglobin specifically labeled at the C-2 histidine position. Measurement of the spin lattice relaxation times of the label before and after hemolysis of the erythrocytes provides information on the intracellular fluid viscosities.*

Suspensions of some cells (*1, 2*) and even tissues (*3*) exhibit ^{13}C nuclear magnetic resonance (NMR) spectra characterized by a high degree of resolution, thus providing an opportunity for studies of the structure and dynamics of molecular constituents in intact cells and tissues. In complex systems containing large numbers of carbon atoms, selective enrichment with ^{13}C offers a number of advantages (*4*), and the introduction of the isotope label does not perturb (*5*) the experimental system to the extent that fluorescent probes or paramagnetic spin labels do. In this study we have used

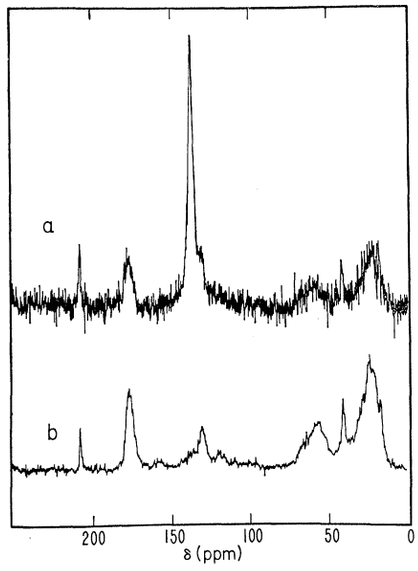

Fig. 1. (a) Carbon-13 Fourier transform NMR spectra of [^{13}C]histidine-labeled carbonmonoxyhemoglobin; (b) natural abundance mouse carbonmonoxyhemoglobin. The spectrum in (a) represents 8,106 pulses; the spectrum in (b) represents 68,700 pulses. The samples contained different concentrations. The spectra were taken at 25°C.

^{13}C spin lattice relaxation time (T_1) measurements to demonstrate that the rotational mobilities of intracellular and extracellular mouse hemoglobin labeled with [^{13}C]histidine differ only by ~25 percent. This result suggests that the viscosity of the intracellular fluid is not "unusually" large (at least in the erythrocytes studied here), a question that has been the subject of recent discussions (*6, 7*).

The ^{13}C-labeled erythrocytes and hemoglobin were obtained from 12 mice [Los Alamos inbred strain, RF(H^2K)] fed freely for a minimum of 65 days on a histidine-deficient diet (General Biochemical) supplemented with 0.2 percent by weight of L-[2-^{13}C]histidine (90 atom percent ^{13}C). The histidine was synthesized by A. Murray according to the method of Tesar and Rittenberg (*8*) and Ashley and Harrington (*9*). The Fourier transform (FT) ^{13}C NMR spectra were obtained at 25.2 Mhz with a Varian XL-100 spectrometer interfaced to a Supernova computer and locked to the deuterium resonance (15.4 Mhz) of an external D_2O capillary. Measurements of T_1 were made by using a pulse sequence (180°-*t*-90°, where *t* is a suitable time delay), followed by Fourier transformation of the free induction decay (*10*). The resulting partially relaxed Fourier transform (PRFT) spectra can be plotted as a function of *t* to obtain T_1.

A spectrum of mouse hemoglobin labeled with [^{13}C]histidine is shown in Fig. 1a, and the spectrum of unlabeled mouse hemoglobin is shown in Fig. 1b. The intense resonance centered at 135.8 parts per million (ppm) (with respect to external tetramethylsilane) in Fig. 1a corresponds to the resonances at the 2-carbon of each of the 40 histidine residues in mouse hemoglobin (*11*), the chemical shifts of the center of the envelope corresponding closely to the histidine C-2 shift in the isolated amino acid (*12*). No resonances outside of this envelope corresponding to the histidines directly coordinated to the iron were observed. Such a resonance would correspond to four histidines per molecule and would be approximately as intense as the observed CO resonance in carbonmonoxyhemoglobin. Since the CO ^{13}C resonance in carbonmonoxyhemoglobin is shifted by only 22 ppm relative to a saturated CO solution (*13*), an even smaller shift would be expected for the histidine ^{13}C-2 not directly coordinated to the iron. The width of the envelope is large, 85 hertz (full width at half maximum intensity), and may reflect small chemical shift inequivalencies of the many histidine residues together with a pronounced broadening of the individual ^{13}C-2 resonances (50 to 60 hertz) because the hemoglobin has a long rotational correlation time, as shown below.

The intensity data obtained from the PRFT spectra of hemoglobin solutions and erythrocyte suspensions at a number of frequencies on the upfield and downfield sides of the ^{13}C-2 maximum at 135.8 ppm as a function of the delay time (*t*) between the 180° and 90° pulses, indicates that most of the ^{13}C-2 resonances are equivalent in their spin lattice relaxation behavior and exhibit an exponential recovery of magnetization. In Fig. 2 are plotted the intensity data (obtained at the 135.8-ppm peak maximum from the PRFT spectra) as a function of *t* for the [2-^{13}C]histidine resonances of a carbonmonoxyhemoglobin solution and an erythrocyte suspension at equivalent hemoglobin concentrations. The average T_1 values for the two systems are 114 msec and 138 msec, respectively. Since T_1 is a double-valued function of the rotational correlation time (τ_R), the measured values do not uniquely determine τ_R. Thus, for the hemoglobin in erythrocyte suspension, τ_R can equal 3.3×10^{-10} second or 4.1×10^{-8} second; and for the hemoglobin in homogeneous solution τ_R can equal 4.1×10^{-10} second or 3.3×10^{-8} second (*14*). Several methods are available for distinguishing the side of the T_1 minimum to which the measurement corresponds. The simplest is a measurement of Overhauser enhancements (*7*). For $(\omega_C + \omega_H)\ \tau_R \ll 1$ (where ω_C and ω_H are the ^{13}C and ^{1}H frequencies), the theoretical enhancement is 2.988, whereas if the rotation is sufficiently slow so that $\omega_C\ \tau_R \gg 1$, the nuclear Overhauser enhancement should decrease to 1.153 (*15*). Overhauser enhancement measurements gave values of 0.99 and 0.96, consistent with the longer values for τ_R. Such correlation times fall within the range expected for a protein the size of hemoglobin and compare favorably with the value of 40 nsec found for the α-carbon atoms of hemoglobin (*16*), which are rigidly locked into the hemoglobin structure. These data suggest that the histidine residues are locked tightly into the hemoglobin structure, and that the correlation time for the relaxation of the ^{13}C-2 resonances is the correlation time for the rotation of the hemoglobin molecule.

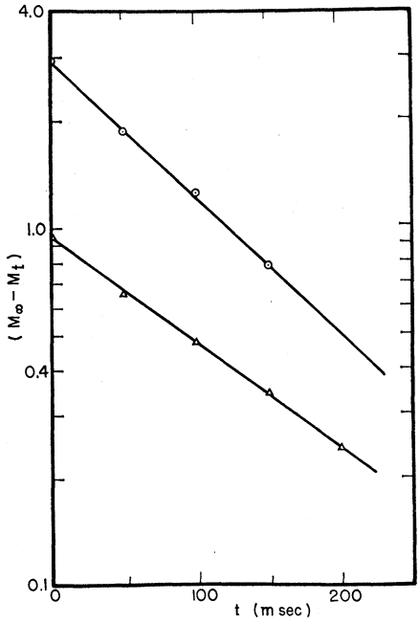

Fig. 2. T_1 plots—$(M_\infty - M_t)$ as a function of *t* (where *M* is magnetization)—for the histidine peak of enriched hemoglobin in whole erythrocytes (△) and hemolyzed, carbonmonoxy-saturated erythrocytes (○) at 23°C.

The absence of any dramatic change in the rotational correlation time accompanying hemolysis suggests that the

intracellular viscosity is not significantly greater than an aqueous solution of the hemoglobin at a similar concentration. Using the Stokes-Einstein equation and a value of 55 Å for the molecular diameter (*17*) gives viscosities of 1.94 centipoise and 1.56 centipoise before and after hemolysis. These results support the view that the protoplasmic viscosity is not very different from that of pure water; most of the differences that do exist can be explained by the high concentration of protein present. The results differ, however, from the general conclusions of Keith and Snipes (*7*) who found large values for the intracellular viscosities of a variety of cells. The difference may reflect differences in the cells tested or in the interpretation of the data, as has been suggested (*6*). In general, the use of ^{13}C NMR to study both intracellular and extracellular proteins should prove valuable for determining differences between the in vivo and in vitro environments of enzymes.

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References and Notes

1. N. A. Matwiyoff and T. E. Needham, *Biochem. Biophys. Res. Commun.* **49**, 1158 (1972).
2. R. T. Eakin, L. O. Morgan, C. T. Gregg, N. A. Matwiyoff, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **28**, 259 (1972).
3. J. D. Robinson, N. M. J. Birdsall, A. G. Lee, J. D. Metcalfe, *Biochemistry* **11**, 2903 (1972).
4. N. A. Matwiyoff and B. F. Burnham, *Ann. N.Y. Acad. Sci.* **206**, 365 (19
5. T. E. Needham, N. A. Matwiyoff, T. Walker, H. P. C. Hogenkamp, *J. Am. Chem. Soc.* **95**, 5019 (1973); D. T. Browne, G. L. Kenyon, E. L. Packer, H. Sternlicht, D. M. Wilson, *Biochem. Biophys. Res. Commun.* **55**, 42 (19
6. *J. Am. Chem. Soc.* **95**, 1316 (1974);
7. M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, J. H. Richards, *Biochemistry* **12**, 4733 (1973); *J. Biol. Chem.* **248**, 8306 (1973); I. M. Chaiken, M. H. Freedman, J. R. Lyerla, J. S. Cohen, *ibid.*, p. 884.
8. N. A. Matwiyoff and D. G. Ott, *Science* **181**, 1125 (1973).
9. E. D. Finch and J. F. Harmon, *ibid.* **186**, 157 (1974).
10. A. D. Keith and W. Snipes, *ibid.* **183**, 666 (1974).
11. C. Tesar and D. Rittenberg, *J. Biol. Chem.* **170**, 35 (1947).
12. J. N. Ashley and C. R. Harrington, *J. Chem. Soc.* (1930), p. 2586.
13. R. L. Vold, J. S. Waugh, M. P. Klein, D. E. Phelps, *J. Chem. Phys.* **48**, 3831 (1968).
14. M. O. Dayhoff, Ed., *Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Washington, D.C., 1969).
15. J. B. Stothers, *Carbon-13 NMR Spectroscopy* (Academic Press, New York, 1972), p. 479.
16. P. Vergamini, private communication.
17. A. Allerhand, D. Doddrell, R. Komorowski, *J. Chem. Phys.* **55**, 189 (1971).
18. D. Doddrell, V. Glushko, A. Allerhand, *ibid.* **56**, 3683 (1972).
19. R. Visscher and F. R. N. Gurd, *Fed. Proc.* **33**, 1441 (1974).
20. M. F. Perutz, *Proc. R. Soc. London Ser. B Biol. Sci.* **173**, 113 (1969).
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Inhibition of Immune Responses in vitro by Specific Antiserums to Ia Antigens

Abstract. *Mouse antiserums prepared against Ia antigens, which are products of I (immune reponse) region genes of the H-2 complex, can inhibit both primary (immunoglobulin M) and secondary (immunoglobulin G) immune responses in vitro by mouse spleen cultures to heterologous erythrocytes. Antiserums directed specifically at products of either the H-2K or H-2D loci have no effect on this response.*

Recently we and others have described a system of antigens, Ia, which is controlled by the I (immune response) region of the H-2 gene complex (*1–4*). These antigens are expressed only on a subset of lymphocytes, which led to early conclusions that Ia antigens were confined to either thymus-derived (T) cells (*3, 4*), or bone marrow–derived (B) cells (*2*). We have shown that Ia antigens are expressed both on T and B cells (*5*).

If Ia antigens are related either to *Ir* gene products or to the cooperation factors postulated by Katz (*6*), then one might predict inhibition of immune response in vitro by antiserum to Ia antigens, by direct blockage of either the antigen receptor or of the cooperation sites on the cell membrane by antibodies in the serum. Pierce *et al.* (*7*) have shown that polyspecific H-2 alloantiserums, which could contain antibodies to Ia, inhibit primary responses in vitro to sheep red blood cells (SRBC).

Using carefully defined antiserum to Ia, antiserum to H-2K, or antiserum to H-2D, we have been able to show inhibition of the secondary response to burro red blood cells (BRBC) in vitro, as well as inhibition of the primary response to SRBC.

All mice used were raised in the mouse colony of the Department of Human Genetics, University of Michigan. Antiserums specific for Ia antigens were produced by reciprocal immunization of A.TH and A.TL mice (*1*). These strains are identical in their H-2K and H-2D regions, and differ only in the I and S (serum protein) regions of the H-2 complex. All serums used came from a single pool that was well characterized for cytotoxic activity. These serums contain multiple antibodies to antigenic determinants mapping in the I region. Antiserum to $H\text{-}2K^s$ was prepared in $(A \times A.AL)F_1$ mice by immunization with tissue from A.TL mice. Two antiserums reacting with H-2D specificities were used as controls: (i) $(C3H \times B10)F_1$ antiserum to C3H.Q, an antiserum to H-2.13, which might contain antibodies to I^q region antigens but could not contain antibody to I^k region antibodies because the $H\text{-}2^k$ haplotype is present in the recipient; or (ii) $(B10 \times AKR.M)F_1$ antiserum to B10.A, which could also contain antibody to I^d region antigens but also is blocked for I^k region antigens by the presence of AKR.M in the recipient. Antiserums were diluted 1 : 2 and sterilized by filtration before they were used in cultures.

Mouse spleen cell cultures were established by a modification of the method of Mishell and Dutton (*8*). Primary cultures were made with SRBC from a single animal and assayed on day 4 for direct plaque-forming cells (PFC) by a modification of the method of Jerne and Nordin (*9*). For secondary responses, spleen donors were immunized at least 3 weeks before use with 0.1 ml of a 10 percent suspension of BRBC. Cultures were stimulated with BRBC and assayed on day 4 for both direct and indirect PFC. For cultures treated with antiserum, two procedures were used. In the primary responses, 100 μl of the sterile diluted antiserum was added to the cultures on day 0. In the secondary responses, spleen cells were treated before culture with a single concentration of antiserum (usually 1 : 2) for 20 minutes at room temperatures, and then placed on ice for 20 minutes. The cells were washed four times and then cultured with BRBC.

After adding either of the two specific antiserums to Ia to unprimed cell cultures we observed a depression of the number of direct PFC recovered. However, in many experiments there were suppressive effects of normal serum, which did not seem to be related to a particular serum, since the same pool of normal serum might be suppressive one day and inactive another. We have pooled the data from the experiments on primary responses from all treatments at concentrations of serum greater than 1 : 1000. Responses of cultures treated with nor-