Phosphorus-31 Nuclear Magnetic Resonance Probes for the Combining Site of the Myeloma Protein M315†

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ABSTRACT: The depth and charge distribution in the combining site of the nitrophenyl binding mouse (IgA, λ_2) myeloma protein M315 are investigated by using ^{31}P NMR probes. The pK_a changes of phosphonate and phosphate haptens, on binding to the Fv fragment of M315, are compared. The pK_a values of *N*-(dinitrophenyl)- [or -(trinitrophenyl)-] 3-aminopropyl phosphonates decrease by 0.7 (and 0.6) unit on binding to the protein. These data are consistent with the interpretation of a positively charged residue located ca. 0.9 nm from the nitrophenyl ring subsite of M315 at the entrance of the combining site and previously assigned to Arg-95 on the light chain. By contrast, the pK_a value of *N*-(dinitrophenyl)-2-aminoethyl phosphate increases by 0.3 unit while that of the corresponding Tnp derivative is essentially unchanged. The difference in behavior between these phosphate and phosphonate haptens, which are all of identical overall length, is interpreted in terms of a solvation charge contribution to the pK_a perturbation of the phosphate haptens. It is suggested that phosphonate derivatives will more accurately reflect the charge distribution in protein combining sites. The series of *N*-(dinitrophenyl)-2-aminoethyl (phosphate)$_n$, n = 1–3, has been used to probe the depth of the M315 combining site. The binding constants, pK_a values, ^{31}P NMR line-broadening data, and the unchanged values of the ^{31}P–O–^{31}P coupling constants indicate that only the α-phosphate group of these haptens interacts with the protein. This gives the depth of the site as ca. 1.0 nm in good agreement with the results from other techniques. The line width of the α-phosphate ^{31}P resonance can be accounted for by a chemical shift anisotropy relaxation mechanism with a value for the anisotropy of ca. 500 ppm. This value may result from an electronic distortion of the phosphate environment caused by the proximity of Arg-95 on the light chain.

X-ray crystallographic analyses have shown that immunoglobulin molecules are composed of several domains, all of which have very similar tertiary structures (Poljak et al., 1972; Schiffer et al., 1973; Segal et al., 1973; Amzel et al., 1974; Epp et al., 1975; Huber et al., 1976). This basic structural unit has been termed the immunoglobulin fold (Poljak, 1975). Comparison of X-ray structures shows that in the variable domains, the immunoglobulin fold acts as a rigid framework to which the hypervariable regions that form the antibody combining site are attached.

The number of different antibody molecules an individual can produce, even against a single antigenic determinant, is very large (Kreth & Williamson, 1973; Pink & Askonas, 1974). It therefore follows that it is not feasible on a reasonable time scale to solve the structures of a sufficient number of antibody combining sites by X-ray crystallographic methods, to provide a broad data base for an understanding of the molecular basis of immune recognition. Several attempts have thus been made to use the crystallographic data presently available and the sequence homologies between antibodies of variable domains (Kabat et al., 1976) to predict the structures of antibody combining sites (Potter, 1977; Kabat, 1978). The first quantitative structure prediction attempt for an antibody combining site was made for the Dnp-binding mouse myeloma (IgA, λ_2)[1] protein M315 by Padlan et al. (1976). This structure has been used by Dwek et al. (1977) as a basis for an extensive series of physical studies. These have led to an understanding of the molecular basis of the specificity of this protein for Dnp derivatives and related aromatic ligands (Dower et al., 1977, 1978; Wain-Hobson et al., 1977; Sutton et al., 1977; Gettins et al., 1978; Morris et al., 1978).

An important part of the approach used in these studies relied upon the use of the immunodominant Dnp or Tnp group as a means of carrying NMR probes into the M315 combining site. For example, the electron spin resonance studies of Sutton et al. (1977) gave information about the depth, rigidity, asymmetry, and polarity of the site. These studies confirmed the presence of a positively charged residue identified as Arg-95$_L$ at the entrance to the combining site of M315, as previously demonstrated by kinetic methods (Haselkorn et al., 1974).

In this paper we report the use of ^{31}P NMR probes to characterize the combining of protein 315. These probes include phosphate haptens, phosphonate haptens, and a series of oligophosphate derivatives *N*-(dinitrophenyl)-2-aminoethyl (phosphate)$_n$ (n = 1–3). It is shown that a marked difference in behavior exists between phosphate and phosphonate derivatives in this system. Phosphate derivatives show a large solvation contribution to the pK_a perturbation while phosphonate derivatives do not. It is concluded that phosphonate derivatives more accurately reflect charge interactions in protein combining sites. The oligophosphate haptens are also used to explore the depth of the combining site.

Materials and Methods

(a) Preparation of N-(Dinitrophenyl)-2-aminoethyl Phosphates. (1) Preparation of N-(Dinitrophenyl)-2-aminoethyl Phosphate. *O*-Phosphorylethanol-2-amine (3.5 mmol, 0.5 g) (Calibiochem, Hereford, U.K.) was reacted with 2,4-dinitrofluorobenzene (3.25 mmol, 0.6 g) (Aldrich Chemicals, Gillingham, Dorset) in 16 mL of 0.2 M $NaHCO_3$. Dinitrofluorobenzene dissolved in 12 mL of dioxane was added dropwise over a period of 1 h to a solution of *O*-phosphorylethanol-2-amine at 37 °C. The reaction was continued until the solution became clear.

The pH of the solution was adjusted to 1 with concentrated hydrochloric acid, and the acid solution was then extracted rapidly with 5 × 1 volumes of ethyl acetate. The ethyl acetate

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[1] Abbreviations used: IgA, immunoglobulin A; Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl; NMR, nuclear magnetic resonance; Fv fragment, variable heavy (H) plus variable light (L) domains of an immunoglobulin molecule; DEAE, diethylaminoethyl.

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Table I: ^{1}H and ^{31}P NMR Data for 2,4-(Dinitrophenyl)-2-aminoethyl Phosphate Derivatives

	^{1}H Chemical Shifts (ppm)[a]				
compd[c]	H_3	H_5	H_6	1 CH	2 CH
I	9.17	8.36	7.28	3.85	4.1
II	9.16	8.36	7.31	3.87	4.24
III	9.17	8.37	7.31	3.89	4.30

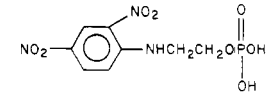
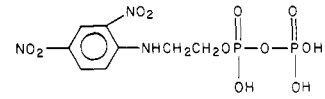
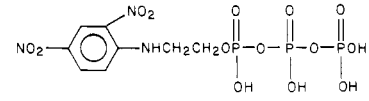
	^{1}H Coupling Constants (Hz)			
compd[c]	$J_{H_3H_5}$	$J_{H_5H_6}$	J_{H_1P}	$J_{H_1H_2}$
I	NS[d]	9.8	5.4	5.4
II	NS	9.8	NS	NS
III	NS	9.8	7.8	4.9

	^{31}P Chemical Shifts (ppm)[b]		
compd	α	β	γ
I	0.75		
II	10.69	9.95	
III	10.59	22.33	10.15

	^{31}P-O-^{31}P Coupling Constants (Hz)		
compd	$J_{\alpha\beta}$	$J_{\beta\gamma}$	pH
II	20		5.4
	22		8.27
III	19	19	5.29
	20	20	8.18

[a] ^{1}H chemical shifts were measured at 270 MHz from DSS as an external standard. All samples were 2 mM in $^{2}H_2O$, pH* 7.1, and 0.15 M NaCl, at 303 K. [b] ^{31}P chemical shifts were measured at 36.43 MHz from 85% phosphoric acid as an external standard. All samples were 5 mM, in $^{2}H_2O$-$^{1}H_2O$, 1:4, pH 5.0, 0.15 M NaCl, and 80 mM EDTA, at 303 K. [c] For formulas refer to Table II. [d] NS represents no coupling observed.

Table II: Molar Absorbances of *N*-(Dinitrophenyl)-2-aminoethyl Phosphates

hapten	E_{360}[a]
 I, *N*-(dinitrophenyl)-2-aminoethyl phosphate	17.2×10^3
 II, *N*-(dinitrophenyl)-2-aminoethyl diphosphate	16.7×10^3
 III, *N*-(dinitrophenyl)-2-aminoethyl triphosphate	17×10^3

[a] Absorbances given are for a 1 M solution, pH 7.0, 1.5 M NaCl, in 50 mM Pipes buffer at 297 K and 360 nm.

solution was dried over sodium sulfate, and the ethyl acetate was removed by rotary evaporation. The yellow solid was redissolved in a minimum volume of water and lyophilized overnight. Yield was 0.89 g, 90% initial Dnp. Purity was checked by ^{1}H NMR and ^{31}P NMR (Table I) and by thin-layer chromatography on polyamide sheets (B.D.H. Chemicals, Poole, Dorset) in 1:2:17 0.880 M ammonia–water–propan-2-ol, in which the R_f value was 0.74. Anal. Calcd for $C_8H_{10}N_3O_8P$: C, 31.3; H, 3.25; N, 13.7; P, 10.1. Found: C, 31.2; H, 3.27; N, 13.5; P, 9.9.

(*2*) *Preparation of N-(Dinitrophenyl)-2-aminoethyl Diphosphate and N-(Dinitrophenyl)-2-aminoethyl Triphosphate.* The method is based on a preparation of adenosine 5-triphosphate by Smith & Khorana (1958). *N*-(Dinitrophenyl)-2-aminoethyl phosphate (2 mmol, 0.614 g) (prepared as above) was condensed with orthophosphoric acid (20 mmol, 2.32 g) by using dicyclohexylcarbodiimide (100 mmol, 20.6 g) (Aldrich Chemicals, Gillingham, Dorset), for 40–50 h at 20 °C. After reaction, the pyridine solution was filtered to remove dicyclohexylurea, which was washed with water. The aqueous filtrate and washings were extracted with 3 × 70 mL of diethyl ether to remove pyridine, and the aqueous phase was made up to 100 mL with water. The ether washings were discarded and the aqueous solution was lyophilized overnight. The yellow solid was redissolved in water (100 mL) and allowed to stand overnight to hydrolyze any residual dicyclohexylcarbodiimide (recovery: 1.97 mmol of Dnp, 99% initial Dnp). The solution was filtered and applied to a 2.8 × 17 cm Poropak Q column, prepared for use by washing with acetone, ethanol, and water (Niederwieser, 1971). Inorganic phosphates were eluted with water, and the effluent was tested for phosphate by ammonium molybdate precipitation. When no phosphate was present in the effluent, the organic phosphates were eluted with 4:1 acetone–water. Acetone was removed from the eluant by rotary evaporation, and the aqueous residue was made up to 50 mL with water (total fraction of initial Dnp recovered: 1.64 mmol, 82%). The solution was applied to a 3.3 × 35 cm DEAE-Sephadex A-25 column. The Dnp phosphates were separated with a 2-L 0.0–1.5 M ammonium bicarbonate (pH 7.8) gradient at a flow rate of 100 mL/h (Figure 1a). The fractions indicated were pooled, desalted by using the Poropak Q column, and, after removing acetone, lyophilized overnight to remove any residual ammonium bicarbonate. The yellow solid (constituting 1.04 mmol of Dnp, 52% initial) was redissolved in 50 mL of 0.1 M ammonium bicarbonate, applied to the DEAE-Sephadex column, and eluted with a 4-L 0.1–0.6 M ammonium bicarbonate gradient (Figure 1b), at a flow rate of 100 mL/h. Fractions were pooled as indicated, desalted by using the Poropak Q column procedure, and lyophilized. Yield: *N*-(dinitrophenyl)-2-aminoethyl diphosphate, 0.14 g, 16% initial; *N*-(dinitrophenyl)-2-aminoethyl triphosphate, 0.26 g, 24% initial. Anal. Calcd for triammonium *N*-(dinitrophenyl)-2-aminoethyl diphosphate monohydrate ($C_8H_{22}N_6O_{12}P_2$): C, 21.04; H, 4.82; N, 18.4; P, 13.6. Found: C, 21.04; H, 4.79; N, 17.7; P, 13.5. Anal. Calcd for tetraammonium *N*-(dinitrophenyl)-2-aminoethyl triphosphate dihydrate ($C_8H_{26}N_7O_{15}P_3$): C, 16.8; H, 4.6; N, 17.1; P, 16.3. Found: C, 16.8; H, 4.7; N, 16.8; P, 16.5. The purity was checked by thin-layer chromatography as described above. R_f values: diphosphate, 0.56; triphosphate, 0.22. ^{1}H NMR and ^{31}P NMR data were also obtained (Table I). The absorbances of these compounds at 360 nm have been determined (Table II).

(*3*) *N-(Dinitrophenyl)-3-aminopropyl phosphonate* was prepared by the same method as *N*-(dinitrophenyl)aminomethyl phosphonate, described in Wain-Hobson et al. (1977), starting from 2,4-dinitrofluorobenzene (0.9 mmol) and (3-aminopropyl)phosphonic acid (1 mmol).

N-(Trinitrophenyl)-2-aminoethyl phosphate was prepared by the same method as *N*-(dinitrophenyl)-2-aminoethyl phosphate.

N-(Trinitrophenyl)-3-aminopropyl phosphonate and *N*-(trinitrophenyl)-2-aminoethyl phosphonate were prepared as described in Wain-Hobson et al. (1977).

(*b*) *Preparation of Solutions of the Fv Fragment of Protein 315.* The Fv fragment of M315 was prepared as described by Inbar et al. (1972). Samples for the NMR studies were prepared by freeze drying the protein from $^{2}H_2O$ (Ryvan Chemicals, Hedge End, Southampton). The pH* (pH un-

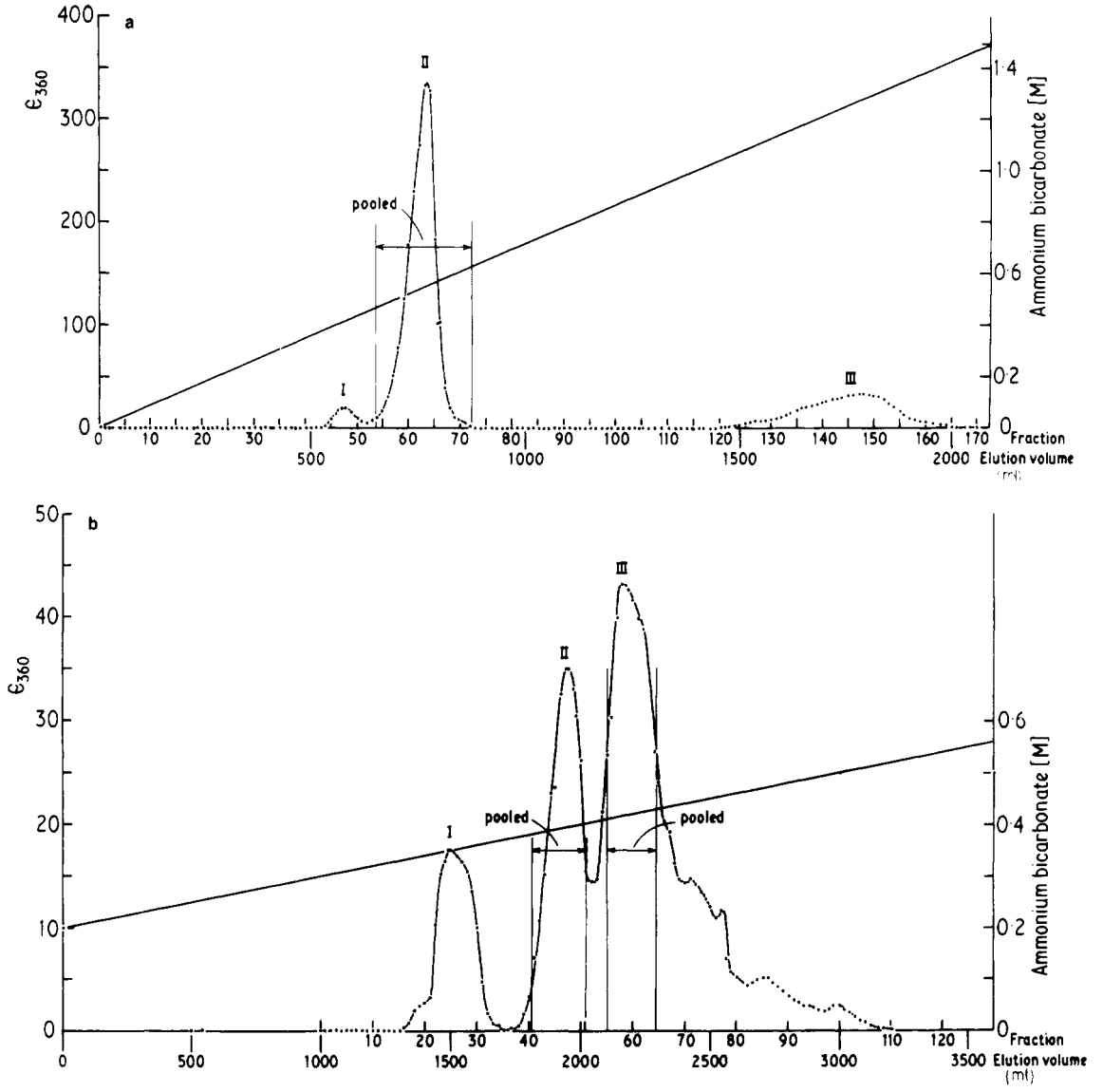


FIGURE 1: Purification of *N*-(dinitrophenyl)-2-aminoethanol derivatives. (a) First ion-exchange step. The peaks are identified as follows: I is *N*-(dinitrophenyl)-2-aminoethyl phosphate. II is a mixture of *N*-(dinitrophenyl)-2-aminoethyl oligophosphates. III is bis[*N*-(dinitrophenyl)-2-aminoethyl] pyrophosphate. (b) Rechromatography of fraction II. The peaks are identified as follows: I is *N*-(dinitrophenyl)ethyl phosphate. II is *N*-(dinitrophenyl)-2-aminoethyl diphosphate. III is *N*-(dinitrophenyl)-2-aminoethyl triphosphate. Conditions are as described in the text.

corrected for the ^2H isotope effect) was adjusted with dilute solutions of ^2H_2O and NaO^2H. All solutions contained 0.13 M NaCl.

(*c*) *Fluorescence Studies.* Fluorescence spectra were recorded on a Perkin-Elmer MPF-2A spectrofluorometer as described by Dwek et al. (1976).

(*d*) *^{31}P NMR Studies.* ^{31}P NMR spectra were recorded at 36.43 MHz on a Bruker WH-90 spectrometer, operating in the Fourier transform mode. The field frequency lock was obtained by using the sample solvent ^2H resonance. Samples were 1.5 mL, in a 5 mM tube fitted with a Teflon vortex plug. All spectra were collected with a spinning sample. In all cases data were collected with proton decoupling.

The pH was adjusted as described above, and measurements were uncorrected meter readings in 1:4 ^2H_2O–^1H_2O from a radiometer pH meter with a glass electrode. pH was measured before and after a spectrum was collected.

In all spectra a 60° pulse angle was used. For free hapten, spectra pulse repetition rates in the range 1–4 s were used; for protein-bound spectra a pulse repetition rate of 0.5–1 s was used.

(*e*) *Analysis of pH Titration Data.* pH titration data from either ^1H NMR or ^{31}P NMR studies are in the form of a chemical shift of one or more resonances as a function of pH. If a single resonance is seen to change shift continuously in this fashion, then the pK_a that the resonance shift change is reflecting can be obtained. The shift (δ_{obsd}) at any given pH will be

$$\delta_{obsd} = \delta_{HA}\bar{x}_{HA} + \delta_{A^-}\bar{x}_{A^-} \quad (1)$$

when δ_{HA} and $\bar{x}_{HA}$ are the chemical shift of and fraction of the protonated form of the system and δ_{A^-} and $\bar{x}_{A^-}$ are similarly defined for the unprotonated form. Equation 1 can be rewritten as

$$\frac{\Delta\delta_{obsd}}{\Delta\delta_0} = \frac{[HA]}{[HA] + [A^-]} \quad (2)$$

when

$$\frac{\Delta\delta_{obsd}}{\Delta\delta_0} = \frac{\delta_{obsd} - \delta_{A^-}}{\delta_{HA} - \delta_{A^-}} \quad (3)$$

Equation 2 can be rewritten as

$$\frac{\Delta\delta_{obsd}}{\Delta\delta_0 - \Delta\delta_{obsd}} = \frac{[HA]}{[A^-]} \quad (4)$$

This can then be substituted into the Henderson–Hasselbalch equation to give

$$pH = pK_a + \log\left(\frac{\Delta\delta_{obsd}}{\Delta\delta_0 - \Delta\delta_{obsd}}\right) \quad (5)$$

If log F, when F is the term in parentheses in eq 5, is plotted as the ordinate, against pH, eq 5 predicts a straight line of slope 1, which intercepts the x axis at pH = pK_a.

To confirm the values for pK_a and $\Delta\delta_0$ measured from the experimental curves, we constructed theoretical curves using HHCAL, a simple Fortran program written in this laboratory.

Results and Discussion

(*a*) *Binding Studies.* The dissociation constants of the haptens were determined by the quenching of protein fluorescence (Table III). All the haptens bind sufficiently strongly to the Fv fragment for the concentration of free hapten to be negligible under the conditions of the ^{31}P NMR experiments.

It is particularly striking that for the three *N*-(dinitrophenyl)-2-aminoethanol derivatives the dissociation constants are very similar. This is consistent with the conclusions from ^{31}P NMR (see section 3b) ^{1}H NMR studies (Dower & Dwek, 1979) that the β- and γ-phosphate groups of these haptens do not interact with the protein.

(*b*) *Comparison of the Effect on the pK_a Values of Phosphates and Phosphonates on Binding to the Fv Fragment of M315.* Data from the ^{31}P NMR probe studies of Wain-Hobson et al. (1977) are given in Table IV. These data show that, on binding to the Fv fragment of M315, the pK_a of the second ionization of *N*-(trinitrophenyl)-3-aminopropyl phosphonate is perturbed downward 0.6 pH unit, while the pK_a values of the two shorter (trinitrophenyl)alkyl phosphonates show small upward perturbations. These data were interpreted as showing that a positively charged residue was located ca. 9.0 Å from the nitrophenyl ring subsite of M315, close to the entrance of the combining site. This conclusion supported that of Haselkorn et al. (1974). It was also observed that the pK_a of *N*-(dinitrophenyl)-2-aminoethyl phosphate was perturbed upward. Since this hapten is of the same extended length as *N*-(trinitrophenyl)-3-aminopropyl phosphonate (Kraut, 1961; Okaya, 1966), this difference in the behavior of two haptens was unexpected. Wain-Hobson et al. (1977) suggested that the difference in behavior was due to the different internal conformations about the nitrophenyl–NH bond in the two haptens, caused by the nitro group in the 6 position of the Tnp ring. This would cause the phosphate group of *N*-(dinitrophenyl)-2-aminoethyl phosphate to be in a different region of the M315 combining site from that occupied by the phosphonate group of *N*-(trinitrophenyl)-3-aminopropyl phosphonate.

The pK_a perturbations observed with *N*-(trinitrophenyl)-2-aminoethyl phosphate and *N*-(dinitrophenyl)-3-aminopropyl phosphonate (Table IV) do not support the above hypothesis. Comparison of the corresponding Dnp and Tnp derivatives of these haptens (Table IV), all four of which are of identical overall length, shows that the pK_a perturbation on binding to the Fv fragment is a function of the chemical nature of the phosphorus derivative and not of the structure of the immunodominant oligonitrophenyl group. The difference in pK_a perturbations is explicable in terms of observations by Crofts & Kosolapoff (1953) and Kumler & Eiler (1943) summarized in Table V. It can be seen that substitution of a methyl group for an ionizable hydrogen in phosphoric acid leads to a decrease in the pK_a of the second ionization for the remaining hydroxyl groups. This effect is in the opposite direction to that expected

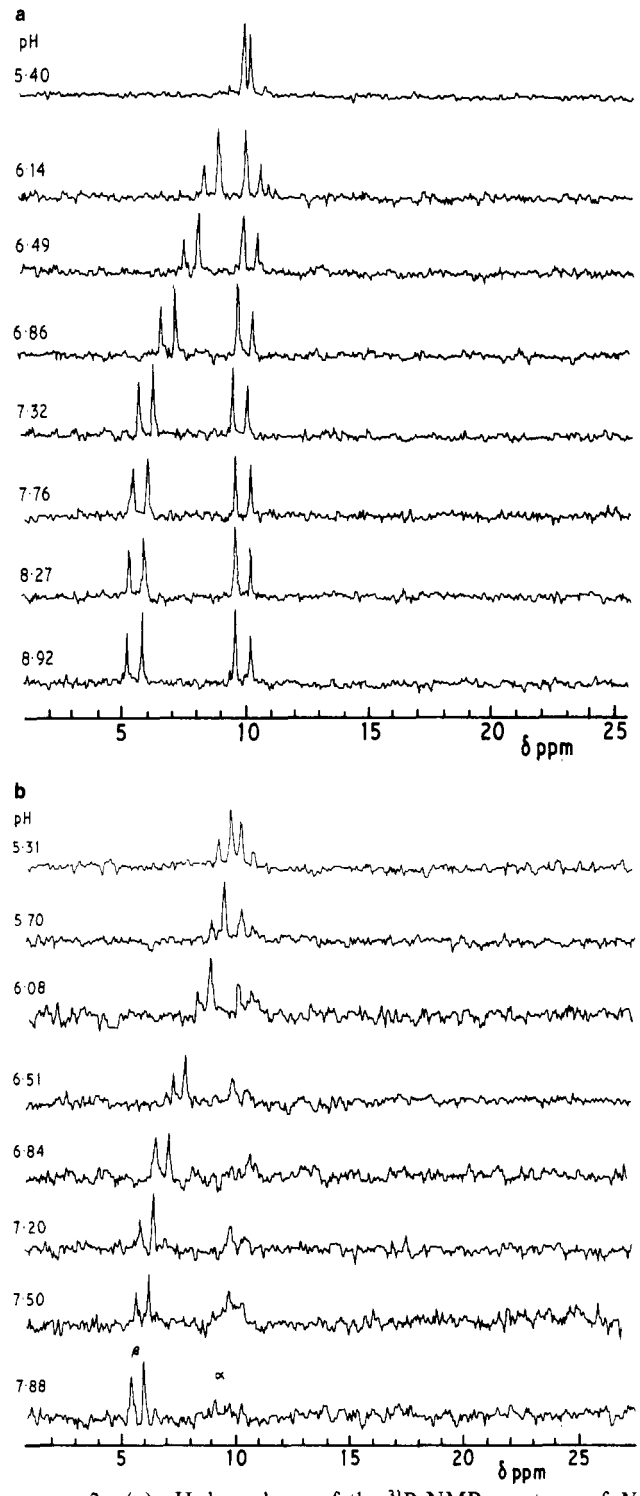


FIGURE 2: (a) pH dependence of the ^{31}P NMR spectrum of *N*-(dinitrophenyl)-2-aminoethyl diphosphate. The sample was 5 mM *N*-(dinitrophenyl)-2-aminoethyl diphosphate, 80 mM EDTA, and 0.15 M NaCl in 1:4 ^2H_2O–^1H_2O; T = 303 K; 1 Hz; 200 scans accumulated per spectrum. (b) pH dependence of the ^{31}P NMR spectrum of *N*-(dinitrophenyl)-2-aminoethyl diphosphate in the presence of the Fv fragment of protein 315. The sample was 0.95 mM *N*-(dinitrophenyl)-2-aminoethyl diphosphate, 1.03 mM Fv fragment, 80 mM EDTA, and 0.15 M NaCl in 1:4 ^2H_2O–^1H_2O; T = 303 K; 1000 scans accumulated per spectrum.

Table III: Dissociation Constants of Haptens from the Fv Fragment of Protein 315[a]

hapten	K_D (μM)
N-(trinitrophenyl)aminomethyl phosphonate	11.0
N-(trinitrophenyl)-2-aminoethyl phosphonate	0.5
N-(trinitrophenyl)-3-aminopropyl phosphonate	0.4
N-(dinitrophenyl)-2-aminoethyl phosphate[b]	0.9
N-(dinitrophenyl)-2-aminoethyl diphosphate[b]	0.62
N-(dinitrophenyl)-2-aminoethyl triphosphate[b]	0.5
N-(trinitrophenyl)-2-aminoethyl phosphate	0.8
N-(dinitrophenyl)-3-aminopropyl phosphonate	0.2

[a] Fluorescence quenching titrations were carried out at pH 5.5 in the presence of 0.15 M NaCl, in 50 mM Pipes buffer, and at 303 K. [b] All conditions were as described above for these experiments except that the pH was 7. The K_D determined for *N*-(dinitrophenyl)-2-aminoethyl phosphate at pH 5.5 was 0.6 μM.

Table IV: pK_a Values for a Set of Phosphate and Phosphonate Haptens Free (f) and Bound (b) to the Fv fragment of Protein 315[a]

hapten	pK_a[f]	pK_a[b]	ΔpK_a
N-(trinitrophenyl)aminomethyl phosphonate[b]	5.7	5.9 ± 0.2	0.2 ± 0.2
N-(trinitrophenyl)-2-aminoethyl phosphonate[b]	6.9	7.1 ± 0.2	0.2 ± 0.2
N-(dinitrophenyl)-2-aminoethyl phosphate[b]	6.3	6.6	+0.3
N-(trinitrophenyl)-2-aminoethyl phosphate	6.2	6.2 ± 0.2	0 ± 0.2
N-(dinitrophenyl)-3-aminopropyl phosphonate	7.55	6.85	−0.7
N-(trinitrophenyl)-3-aminopropyl phosphonate[b]	7.45	6.85	−0.6

[a] All experiments were carried out in 80 mM EDTA and 0.15 M NaCl; T = 303 K. The free-hapten measurements were carried out on a 10 mM solution. The bound-hapten measurements were carried out on 0.9 mM Fv fragment + 0.8 mM hapten. For the free haptens, 200 scans, at a repetition rate of 1.024 s, were accumulated. For the bound haptens, 1000 scans, at a repetition rate of 0.512 s, were accumulated. [b] Data from Wain-Hobson et al. (1977).

Table V: pK_a Values of Alkyl Phosphates and Alkyl Phosphonates in Aqueous Solution[a]

alkyl phosphates[b]		alkyl phosphonates[c]	
derivative	pK_a	derivative	pK_a
phosphoric acid	6.82	phosphorous acid	6.59
methyl phosphate	6.31	methyl phosphonate	7.74
ethyl phosphate	6.62	ethyl phosphonate	8.05
propyl phosphate	6.87	propyl phosphonate	8.18
butyl phosphate	6.84	butyl phosphonate	8.19

[a] The pK_a values given are for the second ionization of the compounds. [b] Data of Kumler & Eiler (1943). [c] Data of Crofts & Kosolapoff (1953).

from the electron donating capacity of alkyl groups. The remaining pK_a changes in the homologous series of alkyl phosphates are consistent with the expected intramolecular inductive effects. In the case of the alkyl phosphonates, all the pK_a values are consistent with the inductive effects of the alkyl groups, the largest increase, as expected, being for the substitution of a methyl group for the nonionizable hydrogen atoms of phosphorus acid. These data show that some effect other than intramolecular electron distribution contributes to the change in pK_a between phosphoric acid and methyl phosphate. Kumler & Eiler (1943) argue that this is caused by a differential aqueous solvation effect which stabilizes the charged states of alkyl phosphates relative to the un-ionized form, producing a downward contribution to the pK_a of 1.0–1.5 units. This effect is absent in alkyl phosphonates.

If the solvation by water of alkyl phosphates diminishes the pK_a by 1.0–1.5 pH units, this effect would be diminished or lost when the phosphate derivative bound to a protein which is more hydrophobic. This would in turn produce an increase in the pK_a of up to 1.0–1.5 pH units, irrespective of the presence or absence of charged amino acid side chains in the binding site. This hypothesis thus provides a simple and direct explanation for the pattern of pK_a changes shown in Table IV. The phosphonate haptens show a downward pK_a perturbation due to the positively charged side chain of Arg-95$_L$ (Wain-Hobson et al., 1977). In the case of the phosphate derivatives, however, this effect is masked by the changes in solvation of the phosphate group. This explanation of the observed pK_a changes is also consistent with the conclusions of Dower et al. (1978) that M315 does not distinguish between Dnp and Tnp derivatives and that both types of hapten have very similar modes of binding.

These data show that some caution should be exercised in interpreting pK_a changes of phosphate ligands, when these bind to proteins, in terms of the proximity of charged groups. We suggest, therefore, that phosphonate analogues of phosphate ligands are more suitable as probes for charge distribution in protein binding sites since these can substitute well for phosphates (Engel, 1977) and do not show any marked solvation contribution to the pK_a. Thus, the data in Table IV also show that there are no charged amino acid side chains in the combining site of protein 315, between Arg-95$_L$ and the nitrophenyl binding subsite. It would be interesting to compare the effects on the pK_a values of phosphorylcholine and the phosphonate analogue of binding to mouse myeloma proteins of the TEPC15 group, since these constitute a well characterized phosphate ligand binding system (Gettins et al., 1977; Goetze & Richards, 1977, 1978).

(*c*) *Oligophosphate Hapten Probes for the Combining Site of the Fv Fragment of M315.* The data given in Table VI show that binding of *N*-(dinitrophenyl)-2-aminoethyl diphosphate and *N*-(dinitrophenyl)-2-aminoethyl triphosphate produces no detectable change in the pK_a values of the final ionization of either compound. Over the pH range 5.4–8.2, the ^{31}P–O–^{31}P coupling constants of the oligophosphate haptens (Table I) also are unaffected by the presence of the Fv fragment of M315. If, as expected, these coupling constants depend on the rotational angles about the P–O bond (Labotka et al., 1976), then these data indicate that the average con-

Table VI: pK_a Values Free (f) and Bound (b) to the Fv Fragment for *N*-(Dinitrophenyl)-2-aminoethyl Oligophosphates[a]

hapten	pK_a[f]	pK_a[b]	$\Delta\delta_A$	$\Delta\delta_B$	P nucleus
N-(dinitrophenyl)-2-aminoethyl phosphate	6.3	6.6	+0.2	+1.3	α
N-(dinitrophenyl)-2-aminoethyl diphosphate	6.53	6.53	+0.3	0	β
N-(dinitrophenyl)-2-aminoethyl triphosphate	6.53	6.53	0	+0.1	γ

[a] δ_A and δ_B are the chemical shift changes for the ^{31}P resonances at the acid and base extremes of the titration curves. The following were the conditions for the free haptens: 5 mM hapten, 80 mM EDTA, and 0.15 M NaCl in 1:4 ^2H_2O–^1H_2O; T = 303 K; 200 scans accumulated. The following were the conditions for the bound spectra: 0.95 mM hapten, 1.03 mM Fv fragment, 80 mM EDTA, and 0.15 M NaCl in 1:4 ^2H_2O–^1H_2O; T = 303 K; 1000 scans accumulated per spectrum.

formation of the oligophosphate group is unaffected by binding to the protein, in both cases. These data suggest that the β- and γ-phosphate groups of these haptens do not interact with the Fv fragment of M315. This conclusion is supported by the similar dissociation constants for the three *N*-(dinitrophenyl)-2-aminoethanol derivatives and by the similar ^{1}H NMR difference spectra produced on binding of the three haptens to the Fv fragment of M315 (Dower & Dwek, 1979). Thus, the α-phosphate group of these haptens lies at the entrance to the combining site, while the β- and γ-phosphate groups lie outside it.

Figure 2 shows that the α-phosphate group ^{31}P resonance of *N*-(dinitrophenyl)-2-aminoethyl diphosphate experiences an increase in line width from 1 to 8 Hz when this hapten binds to the Fv fragment. No such change is seen for the β-phosphate resonance. This is consistent with the former being inside the site and the latter outside. The dipolar contribution to the line width of the ^{31}P resonance will arise mainly from the two protons on the CH_2 group on the side chain of the hapten. These protons are 0.25 nm from the phosphorus nucleus (Kraut, 1961). The rotational correlation time of the Fv fragment–hapten complex can be estimated as 7 ns from Stokes law. By use of the appropriate equation for dipolar relaxation of unlike spins (Dwek, 1973), the calculated ^{31}P resonance line width is <1 Hz. The increase in line width of the α-phosphate group ^{31}P resonance cannot therefore be accounted for by ^{1}H–^{31}P dipolar broadening. The most probable origin of the effect is chemical shift anisotropy (Carrington & MacLachlan, 1967), which, using the relevant equation, would require the chemical shift anisotropy of the α-phosphate group to be ca. in the order of 500 ppm to account for the line width. This is larger than expected for this compound on the basis of the value for adenosine diphosphate (ca. 150 ppm) and may suggest an electronic distortion of the α-P environment. This could be caused by the proximity of this group to Arg-95_L.

Thus, the ^{31}P NMR data indicate that there is a positively charged amino acid side chain at the entrance to the combining side of the Fv fragment of M315, that there are no charged amino acid side chains further into the combining site, and that the combining site is ca. 10 Å in depth. These conclusions are consistent with those arrived at by other techniques, including electron spin resonance (Sutton et al., 1977), ultracentrifugation (Wilder et al., 1975), rapid reaction kinetics (Haselkorn et al., 1974), and affinity chromatography (Rosenstein & Richards, 1976). Finally, we may also note that these oligophosphate haptens can be used to create a specific metal ion binding site on the M315 Fv fragment–hapten complex. Their use in paramagnetic mapping studies by NMR is currently being developed and will be reported elsewhere (Dower & Dwek, 1979).

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References

Amzel, L. M., Poljak, R. J., Saul, F., Varga, J., & Richards, F. F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1427–1430.

Carrington, A., & MacLachlan, A. D. (1967) *Introduction to Magnetic Resonance with Applications to Chemistry and Chemical Physics*, Harper and Row, New York, Evanston, and London, and John Weatherhill, Tokyo.

Crofts, P. C., & Kosolapoff, G. M. (1953) *J. Am. Chem. Soc. 75*, 3379–3384.

Dower, S. K., & Dwek, R. A. (1979) *Int. J. Biol. Macromol.* (in press).

Dower, S. K., Wain-Hobson, S., Gettins, P., Givol, D., Jackson, W. R. C., Perkins, S. J., Sunderland, C. A., Sutton, B. J., Wright, C. E., & Dwek, R. A. (1977) *Biochem. J. 165*, 207–225.

Dower, S. K., Gettins, P., Jackson, R., & Dwek, R. A. (1978) *Biochem. J. 169*, 179–188.

Dwek, R. A. (1973) *N.M.R. in Biochemistry. Application to Enzyme Systems*, Clarendon Press, Oxford.

Dwek, R. A., Jones, R., MacLaughlin, A. C., Wain-Hobson, S., White, A. I., & Wright, C. E. (1976) *Biochem. J. 155*, 37–53.

Dwek, R. A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S. J., & Givol, D. (1977) *Nature (London) 266*, 31–37.

Engel, P. (1977) *Chem. Rev. 77*, 349–374.

Epp, O., Lattman, E. E., Schiffer, M., Huber, R., & Palm, W. (1975) *Biochemistry 14*, 4943–4952.

Gettins, P., Potter, M., Rudikoff, S., & Dwek, R. A. (1977) *FEBS Lett. 84*, 87–91.

Gettins, P., Givol, D., & Dwek, R. A. (1978) *Biochem. J. 173*, 713–722.

Goetze, A. M., & Richards, J. H. (1977) *Biochemistry 16*, 228–230.

Goetze, A. M., & Richards, J. H. (1978) *Biochemistry 17*, 1733–1738.

Haselkorn, D., Friedman, S., Givol, D., & Pecht, I. (1974) *Biochemistry 13*, 2210–2222.

Huber, R., Diesenhoffer, J., Colman, P. M., Matsushima, M., & Palm, W. (1976) *Nature (London) 264*, 415.

Inbar, D., Hochman, J., & Givol, D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2659–2663.

Kabat, E. A. (1978) *Adv. Protein Chem. 32*, 1–59.

Kabat, E. A., Wu, T. T., & Bilofsky, H. (1976) *Variable Regions of Immunoglobulin Chains*, Medical Computer Systems Inc., Cambridge, MA.

Kraut, J. (1961) *Acta Crystallogr. 14*, 1146–1147.

Kreth, H. W., & Williamson, A. R. (1973) *Eur. J. Immunol. 3*, 141–142.

Kumler, W. D., & Eiler, J. J. (1943) *J. Am. Chem. Soc. 65*, 2355–2361.

Labotka, R. J., Glonek, T., & Myers, T. C. (1976) *J. Am. Chem. Soc. 98*, 3699–3704.

Morris, A. T., Givol, D., & Dwek, R. A. (1978) *Immunochemistry 15*, 519–522.

Niederwieser, A. (1971) *J. Chromatogr. 54*, 215–223.

Okaya, Y. (1966) *Acta Crystallogr. 20*, 712–714.

Padlan, E. A., Davies, D. R., Pecht, I., Givol, D., & Wright, C. E. (1976) *Cold Spring Harbor Symp. Quant. Biol. 41*, 627–639.

Pink, J. R. L., & Askonas, B. A. (1974) *Eur. J. Immunol. 4*, 426–433.

Poljak, R. J. (1975) *Adv. Immunol. 21*, 1–33.

Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B., Phizackerly, R. P., & Saul, F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3305–3320.

Potter, M. (1977) *Adv. Immunol. 25*, 141–155.

Rosenstein, R. W., & Richards, F. F. (1976) *Immunochemistry 13*, 939–942.

Schiffer, M., Girling, R. L., Ely, K. R., & Edmunsdon, A. B. (1973) *Biochemistry 12*, 4620–4631.

Smith, M., & Khorana, H. G. (1958) *J. Am. Chem. Soc. 80*, 1141–1145.
Sutton, B. J., Gettins, P., Givol, D., Marsh, D. M., Wain-Hobson, S., Willan, K. J., & Dwek, R. A. (1977) *Biochem. J. 165*, 177–197.
Wain-Hobson, S., Dower, S. K., Gettins, P., Givol, D., MacLaughlin, A. C., Pecht, I., Sunderland, C. A., & Dwek, R. A. (1977) *Biochem. J. 165*, 227–235.
Wilder, R. L., Green, G., & Schumaker, V. N. (1975) *Immunochemistry 12*, 55–65.

Insulin Binding to Isolated Liver Nuclei from Obese and Lean Mice†

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ABSTRACT: Nuclei isolated from the livers of mice are capable of binding [^{125}I]insulin. A class of high-affinity binding sites having a K_d of 1–2 nM and a capacity of ~2000 insulin molecules/nucleus are present on these nuclei. Removal of nuclear membranes by Triton X-100 treatment of the nuclei reduces or eliminates the high-affinity binding sites. Nuclei prepared from livers of the genetically obese mouse (ob/ob) lack, or have markedly reduced numbers of, the high-affinity binding sites whether or not the obese nuclei have been exposed to Triton X-100. The reduced insulin-binding capacity of the obese nuclei correlates with the reported decreased binding of insulin to plasma membranes prepared from target tissue of these animals. The possible physiological significance of nuclear insulin binding is discussed.

Although the theory that insulin does not enter its target cell has been in doubt for some time (Davidson et al., 1973; Katzen & Vlahakes, 1973; Kolb et al., 1975), the concept of an extracellular site as the primary locale for insulin action has remained prevalent. Yet there is reason to doubt a unitary mechanism of hormone action in which insulin mediates its effects solely through binding to receptors at the cell surface. To date a second messenger, comparable to cyclic AMP, which would relay information from the receptors to the intracellular sites where insulin exerts its effects, such as on RNA synthesis (Pilkis & Salaman, 1972) and enzyme induction (Pilkis, 1970; Reel et al., 1970), has not been identified. In addition, correction of defective insulin receptors does not necessarily restore normal metabolic responses to the hormone (Abraham & Beloff-Chain, 1971; Le Marchand et al., 1977).

Recently, the internalization of insulin has been demonstrated (Schlessinger et al., 1978; Carpentier et al., 1978). In addition, there have been several reports characterizing the binding of insulin to various organelles (Bergeron et al., 1978; Horvat et al., 1975; Kahn, 1976; Posner et al., 1978a,b). The findings described in these studies suggest the possibility of intracellular binding sites in the mechanism of action of this important, but still poorly defined, hormone.

In several experimental models of insulin resistance, including the genetically obese mouse (ob/ob), a reduction in the number of insulin receptors associated with the plasma membranes of target tissues has been observed (Goldfine et al., 1973; Harrison et al., 1976; Kahn et al., 1972; Kern et al., 1975; Olefsky & Reaven, 1974; Soll et al., 1975a,b). The present communication describes the binding of insulin to nuclei isolated from livers of these obese mice and their lean littermates. The reduced binding to the "obese nuclei", which correlates with the decreased plasma membrane binding, suggests that the liver cell nucleus may play a physiological role in determining the state of responsiveness to insulin.

Materials and Methods

Female ob/ob mice, their lean littermates, and C57Bl/6J mice were purchased from the Jackson Laboratory, Bar Harbor, ME. [^{125}I]Insulin (~100 μCi/μg) and Aquasol-2 were purchased from New England Nuclear. [2-^{3}H]AMP (18.5 Ci/mmol), [8-^{14}C]adenosine (59 mCi/mmol), and Triton X-100 were obtained from Amersham Corp. Bovine insulin and synthetic somatostatin were products of Calbiochem. Porcine proinsulin, desoctapeptide insulin, and desalanine insulin were gifts from the Eli Lilly Co. Murine EGF[1] was generously provided by C. Richard Savage of Temple University Medical School, Philadelphia, PA. Insulin radioimmunoassay kits and crystallized glucagon were purchased from Schwarz/Mann. Porcine FSH, ovine prolactin, bovine TSH, synthetic oxytocin, and pancreozymin (Grade II) were all products of Sigma. PEI–cellulose F plates for thin-layer chromatography were obtained from EM Laboratories, Inc. Ready-Solv was purchased from Beckman Instruments. Protein assay kits were purchased from Bio-Rad.

Nuclear Preparation. Mice were sacrificed by cervical dislocation. Nuclei were prepared from mouse livers by using the procedure of Blobel & Potter (1966). Detergent-treated nuclei were prepared by suspending the first 340*g* nuclear pellet in TSM buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$) containing 0.5% Triton X-100 and incubating the nuclei at 0–4 °C for 5–10 min. The nuclei were sedimented at 340*g* for 5 min and suspended in TSC buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, and 3 mM $CaCl_2$). Subsequent procedures for both detergent-treated and untreated nuclei were identical. The final nuclear pellets obtained after sedimentation through the 2.2 M sucrose cushion were washed in TSM buffer and then suspended in a volume of assay buffer (0.25 M sucrose, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 2 mM Na_2EDTA, and 5 mg/mL BSA) such that the concentration of DNA was ~1 mg/mL.

High-Speed Supernatant (S100) and Microsomal Preparation. S100 cytosol fractions and microsomal fractions were prepared from the first 340*g* supernatant obtained during nuclear isolation. This low-speed supernatant was centrifuged at 30000*g* for 30 min. The 30000*g* supernatant was then

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[1] Abbreviations used: EGF, epidermal growth factor; FSH, follicle stimulating hormone; TSH, thyroid stimulating hormone; PEI, polyethylenimine; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin.

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