Frozen solutions of biotinylated glucose-6-phosphate dehydrogenase and fluorescently tagged avidin were exposed to high energy ionizing radiation. Parallel experiments with peroxidase coupled to streptavidin and with biotinylated phycoerythrin were also performed. The loss of function of each compound was analyzed according to target theory. Target analysis revealed that the radiation-sensitive mass associated with the enzymatic activity and that associated with the fluorescent were unchanged by irradiation in the strongly coupled state. Therefore the noncovalent bonds between biotin and avidin do not permit the transfer of radiation-deposited energy in amounts sufficient to destroy the activity of apposing molecule.

Exposure to ionizing radiation causes a degradation of macromolecular structure and a loss of biological activity. When irradiations are performed at very low temperatures the indirect damage due to secondary processes is diminished and the predominant effect is due to radiation interactions directly with the macromolecules. The more limited range of effects in the frozen state permits experimental analysis of some of the mechanisms of radiation damage. Previous reports have shown the appearance of this damage throughout a polypeptide no matter where the initial interaction (primary ionization) had occurred (1-3). In disulfide-linked dimers of ricin, radiation damage was observed in the A chain when the primary ionization occurred in the B chain (4). All these studies establish that damage occurs throughout covalently bound structures, presumably by the transfer of energy previously deposited by radiation.

Results from a large number of radiation studies are consistent with these ideas. However, there have been reports of a single radiation event resulting in both structural and functional damage in several subunits even though the subunits were not joined by a disulfide or other covalent bridge (5-11). Such studies provoke questions about the route and mechanism of energy transfer between polypeptides. The most obvious candidate is that of noncovalent bonding and these interactions are tested in this report.

The strong bonding of avidin to biotin displays a dissociation constant of ~10^{-10} M without the involvement of covalent interactions. Therefore, it constitutes an ideal system in which to test the hypothesis that radiation energy can be transferred across noncovalent bonds.

Both avidin (a homotetramer, A4 x 15.6 kDa (12)) and biotin (244 Da) can be coupled to other molecules without loss of their noncovalent bonding. The covalent binding of antibodies, enzymes, or fluorescent compounds to avidin or biotin creates a new "function" which can be monitored after exposure to ionizing radiation. Therefore, radiation inactivation of a biotinylated enzyme which is strongly bound to a fluorescently tagged avidin were used to calculate a target size for structures on each side of the avidin-biotin bonds. Analogous studies with streptavidin covalently coupled to an enzyme and with biotinylated B-phycoerythrin were also conducted. As in the case of the disulfide-linked ricin molecule (4), a target size corresponding to the sum of both units would imply a transfer of energy across these bonds. In contrast, we find smaller, individual target sizes which indicate the absence of major energy transfer across the strong noncovalent bonds that link avidin to biotin.

**Direct Effects of Radiation on the Avidin-Biotin System**

**ABSENCE OF ENERGY TRANSFER**

Ellis S. Kempner and Jay H. Miller

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**MATERIALS AND METHODS**

Glucose-6-phosphate dehydrogenase (G6PDH) from Baker's yeast (Type IX) was purchased from Sigma. The biotinylated form of glucose-6-phosphate dehydrogenase (bG6PDH), also from Sigma, contains 6.5 mol of biotin/mol of G6PDH. The enzyme is attached directly to the biotin carboxyl group with no spacer. Avidin and avidin covalently coupled to the fluorescent dyes Texas Red (Av-TXR) or fluorescein isothiocyanate (Av-FITC) were obtained from Vector Laboratories (Burlingame CA). In these preparations the molar ratio of fluorescent tag per avidin tetramer was 3.0 for TxR and 3.5 for FITC. Prior to use of the fluorescent compounds the buffer was exchanged by means of Bio-Rad 1ODG desalting columns to Tris buffer (55 mM Tris, pH 7.8, containing 4.5 mM MgCl₂ and 150 mM NaCl). Since a high salt concentration is required to keep Av-FITC in solution, all of the present experiments with Av-TXR and Av-FITC were performed in 150 mM NaCl. In samples to be irradiated, final protein concentration was 2 mg/ml, achieved by addition of iron free human transferrin (Sigma).

Horseradish peroxidase and streptavidin were purchased from Sigma. Streptavidin-peroxidase (SAPO) was obtained from Sigma (molar ratio of 1:2 streptavidin per peroxidase), amide groups on both proteins were coupled through a thiouther linkage attached to a 12-carbon spacer. Biotinylated B-phycoerythrin (bBEP) containing 1-3 biotins/phycoerythrin (linked through the carboxyl group of biotin by a 7-carbon spacer) was purchased from Molecular Probes (Analyzed according to target theory. Target analysis revealed that the radiation-sensitive mass associated with the enzymatic activity and that associated with the fluorescent were unchanged by irradiation in the strongly coupled state. Therefore the noncovalent bonds between biotin and avidin do not permit the transfer of radiation-deposited energy in amounts sufficient to destroy the activity of apposing molecule.

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and high voltage on the phototube were adjusted to give a relative intensity ~1.0 for the control (unirradiated) samples in each experiment.

Fluorescence of TxR and FITC were measured at 596/620 and 495/520 nm excitation/emission, respectively. Fluorescence intensity of these compounds was not diminished in the presence of bG6PDH. Fluorescence measurements of phycoerythrin were performed at 545 nm excitation and 575 nm emission.

Enzyme Assays—G6PDH activity was determined as previously described (13). These preparations averaged 137 units/mg protein. The bG6PDH specific activity was 70 units/mg protein. When bound to Av-TxR or Av-FITC, the activity of bG6PDH was diminished: at 9.1 molar ratio of biotin:avidin the activity was reduced to ~55%; at 3:1, ~13%; and at 1:1, ~11% remained.

Peroxidase activity was measured at 25 °C as described in the Worthington Enzyme Manual (32). Assay solution (3 ml) contained enzyme, 0.86 mM H2O2, 1.17 mM 4-aminophenylhydroxylamine, 79.3 mM phenol, and 0.1 M phosphate buffer, pH 7. Change in optical density at 510 nm was followed.

Irradiation Conditions—Samples of 250 µl were dispensed into 2-ml glass vials. The requirement of a high protein concentration during irradiation (14, 15) was satisfied in the present experiments by avidin, streptavidin, or by transferrin. Frozen samples in sealed glass vials were irradiated at ~135 °C with 13 MeV electrons as described previously (16).

Radiation Target Analysis—Radiation inactivation curves from each experiment were analyzed as described (16). A constrained least squares calculation yielded the slope of the curve from which the target size was determined. In all cases the target size (molecular mass) is reported as average ± S.D. for n > 3.

SDS-PAGE—Polyacrylamide gel electrophoresis was performed on an 11% gel at 100 V for 1.5 h. Protein was visualized with Coomassie Blue R-250 and destained with methanol/acetic acid mixtures.

HPLC—Chromatography was performed on a Gilson isocratic HPLC system with a TSK G3000SWXL column (7.8 X 300 mm). Elution buffer was 50 mM Tris, pH 7.0, containing 150 mM NaCl. Flow rate was 0.5 ml/min.

RESULTS

Glucose-6-phosphate Dehydrogenase, bG6PDH, and Fluorescently Tagged Avidin—In preliminary experiments, the amount of bG6PDH bound by avidin was determined by the change in tryptophan fluorescence (17). An average of three biotins from bG6PDH was observed to be bound per avidin (data not shown), confirming a previous report (18). The fluorescence of Av-TxR and Av-FITC obstructed this measurement.

Radiation inactivation of G6PDH leads to a target size in the range of 100–110 kDa, as has been widely reported from this and other laboratories (13, 19, 20). In the present study we have continued to observe such values. However, in the presence of 150 mM NaCl (required to keep Av-FITC in solution), the enzyme shows increased sensitivity to radiation and displays a target size of 130–150 kDa.

The loss of enzyme activity over at least three decades was an exponential function of radiation dose in all experiments, as typified in Fig. 1. Table I shows that the target size for G6PDH is unaffected by the presence of avidin and/or the fluorescently tagged avidin compounds. The biotinylated target size is covalently coupled to TxR is that of a single subunit of the enzyme. This result is to be expected because of the very small increase in size due to biotininvlation (0.2 kDa). When G6PDH or bG6PDH is irradiated in the presence of avidin (data not shown) or fluorescently tagged avidin (Table I), the target size for enzymatic activity does not increase.

There is only a modest decrease of fluorescence of covalently tagged avidin even after large doses of radiation (Fig. 1). Statistical analyses of a large number of experiments indicates that the loss is an exponential function of radiation exposure, leading to a small target size. The limited degree of inactivation results in appreciable variability among calculated target sizes which is reflected in large error values. Table II shows that the target size for fluorescence of avidin which is covalently coupled to TxR is that of a single subunit of the

Table I

<table>
<thead>
<tr>
<th>Target size (in kilodaltons) associated with enzymatic activity</th>
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<td>Irradiations were performed in the presence of 150 mM NaCl. All samples contained 2 mg of protein/ml. The bG6PDH-Av-TxR and bG6PDH-Av-FITC samples contained 3 mol of biotin/mol avidin tetramer (=0.46 mol of bG6PDH dimer/mol avidin tetramer). Other samples contained G6PDH at this concentration of bG6PDH and transferrin at the concentration of the avidin derivatives.</td>
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<tr>
<td>Target size h kilodaltons) associated with enzymatic activity</td>
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<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>G6PDH</td>
</tr>
<tr>
<td>Av-TxR 12 ± 2</td>
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<tr>
<td>Av-FITC 10 ± 2</td>
</tr>
<tr>
<td>G6PDH + biotin</td>
</tr>
<tr>
<td>Av-TxR 28 ± 3</td>
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<tr>
<td>Av-FITC 28 ± 3</td>
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<tr>
<td>bG6PDH</td>
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<td>Av-TxR 12 ± 3</td>
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G6PDH displays the same radiation sensitivity as the native enzyme. This result is to be expected because of the very small increase in size due to biotininvlation (0.2 kDa). When G6PDH or bG6PDH is irradiated in the presence of avidin (data not shown) or fluorescently tagged avidin (Table I), the target size for enzymatic activity does not increase.
avidin tetramer. When this fluorescently tagged avidin is irradiated in the presence of either G6PDH or bG6PDH, the same target size (a single avidin subunit) is observed as in the absence of the enzyme. The target size for fluorescence of Av-FITC is approximately twice that of the TxR derivative, but is similarly unaffected by the presence of G6PDH or bG6PDH.

The target sizes of the biotinylated enzyme and the fluorescently modified avidin are unchanged in the strongly interacting state, suggesting the absence of major energy transfer. However, the results might be misleading if there were a vast molar excess of either the biotinylated enzyme or the tagged avidin, since the observed target sizes could then reflect only the excess, unbound compound. The above experiments were all performed at a molar ratio of three biotins per avidin because preliminary experiments had indicated this to be the maximal binding ratio. The experiments were repeated at molar ratios of 1:1 and 9:1, keeping constant the total protein in each sample. As shown in Table III, the observed target sizes for both enzyme activity and fluorescence were not affected by changes in the ratio of biotin to avidin.

Since the activity of bG6PDH is reduced in the presence of avidin (see "Materials and Methods") it is still possible that only the free bG6PDH is active and the target size for enzymatic activity always reflects the size of free bG6PDH. The fluorescence intensity of Av-TxR is not diminished in the presence of bG6PDH and therefore cannot be ascribed only to unbound molecules. The target analyses based on fluorescence must represent all the Av-TxR molecules present.

Peroxidase, Phycoerythrin, and Their Derivatives—Enzymatic activity was determined in samples of peroxidase which had been irradiated in buffer with transferrin and as the enzyme coupled to streptavidin (SAPO). All samples showed a simple exponential loss of activity with radiation exposure, as shown in Fig. 2 for SAPO. The calculated target sizes are given in Table IV. The target size calculated for peroxidase activity is 28 kDa. When covalently linked to streptavidin, the target size associated with peroxidase activity remains unchanged. In this coupled protein, radiation energy would be expected to transfer through the covalent bridge, giving a target size of 34 (peroxidase) + 13 (streptavidin) = 47 kDa. Since the observed target is only 30 kDa, the energy transfer must not have occurred, either because the assumed covalent bond does not exist or for more complex reasons (such as unique bonds in the coupling arm which block or divert the energy transfer). The presence of the covalent linkage was tested by denaturing gel electrophoresis (SDS-PAGE) and by HPLC analysis of SAPO. As shown in Fig. 3, only two protein bands were observed on electrophoretograms: one band near

<table>
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<th>Table III</th>
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<tr>
<td><strong>Target sizes in samples containing different biotin/avidin ratios</strong></td>
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<tr>
<td>Data are from three or more independent experiments.</td>
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<table>
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<tr>
<th>Biotin</th>
<th>G6PDH</th>
<th>Fluorescent avidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin</td>
<td>nm/ml</td>
<td>Target</td>
</tr>
<tr>
<td>bG6PDH + Av-TxR</td>
<td>1:1</td>
<td>4.2</td>
</tr>
<tr>
<td>3:1</td>
<td>8.7</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>9:1</td>
<td>13.7</td>
<td>135 ± 24</td>
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</table>

| bG6PDH + Av-FITC | 1:1 | 4.2 | 111 ± 37 | 24 | 23 ± 1 |
| 3:1 | 8.7 | 91 ± 6 | 17 | 21 ± 3 |
| 9:1 | 13.7 | 127 ± 19 | 8.7 | 24 ± 11 |

*There are 6.5 M biotin per M G6PDH in these preparations. Therefore 1:1 mol of biotin moieties per M avidin tetramer = 0.15 mol of bG6PDH dimer/mol avidin tetramer.

FIG. 2. Loss of peroxidase activity in solutions of SAPO irradiated frozen. Data points show average and S.D. from three independent experiments.

FIG. 3. SDS-PAGE. Proteins visualized by Coomassie stain. Standards in left lane are (top to bottom) Mr = 67,000, 43,000, 25,000, and 12,500. Second lane is streptavidin (13.2-kDa subunit). Third lane is SAPO, which resolves into a band of free streptavidin subunits and a band of streptavidin subunits covalently coupled to peroxidase (Mr ≈ 58,000). Right lane contains the glycoprotein peroxidase (Mr = 44,000). No free peroxidase was detected in the SAPO preparation.

Mr = 50,000 which corresponds to peroxidase covalently linked to a streptavidin subunit, and a second band near Mr = 15,000 which corresponds to individual streptavidin subunits. No protein was detected at the position of free peroxidase. On HPLC, peroxidase activity was recovered only at the
position of the covalently linked SAPO. These data are consistent with 1) a covalent bridge between streptavidin and peroxidase and 2) that all detectable activity is associated with this linked molecular species. The unexpectedly small target size of peroxidase must be due to a blockage of the transfer of energy across the thioether, 12-carbon linker.

The loss of phycoerythrin fluorescence was also determined after irradiation in buffer, in the presence of biotin, and as the biotinylated derivative (Fig. 4). The target sizes obtained for each were the same: 29 kDa, 31 kDa, and (the biotinylated fast) 29 kDa.

When the boE-SAPO complex (at 3:1 biotin:streptavidin ratio) was irradiated, a target size of 32 kDa was observed for enzymatic activity and 27 kDa for fluorescence (Table IV).

SAPO and boG6PDH—In parallel experiments, SAPO was irradiated in the presence of biotinylated glucose-6-phosphate dehydrogenase at a biotin:streptavidin molar ratio of 3:1. In three independent experiments the target size for peroxidase activity in this complex was 27.0 ± 2.7 kDa, which is indistinguishable from the target size for peroxidase alone (Table IV) of 28.9 ± 3.4 kDa.

**DISCUSSION**

**G6PDH and Avidin**—Although the target size associated with the enzymatic activity of G6PDH is larger in 150 mM NaCl than in buffer alone, the addition of biotin or biotinylation of the enzyme has no further effect. The effect of NaCl on target size is the subject of a separate study.

Avidin is a homotetrameric glycoprotein. The radiation destruction of fluorescence of tagged avidin molecules is associated with a target size much smaller than the entire structure. TxR fluorescence requires the integrity of only a single subunit polypeptide, a result similar to that previously found in derivatized glutamate dehydrogenase (21). As in that experiment, it can be concluded that there is no massive transfer of radiation-deposited energy between the four avidin subunits.

In boG6PDH, the enzyme is attached to the carboxyl group on biotin. Attachment of macromolecules at this position does not affect the binding of biotin to avidin (12). When the enzyme is irradiated as a boE-streptavidin complex, there is no increase in the boG6PDH target size compared to the enzyme alone. This result suggests that the biotin-avidin interactions are not sufficient to permit the transfer of energy from radiation-damaged avidin to the enzyme. However, since the target size associated with avidin functions is small (~20 kDa) compared to that of the enzyme (~140 kDa), it might be difficult to resolve a potential increase of only 14%. The fluorescence measurements of the avidin-biotin complex are more convincing, since an 8-fold change in target size (from ~20 to ~160 kDa) would be easily detected. The data show unequivocally that primary ionizations in bG6PDH do not result in transfer of energy across the bonds to avidin in amounts sufficient to cause loss of fluorescence.

**Peroxidase and Streptavidin**—The conclusions above were based primarily on measurements of the smaller avidin molecule which is a glycoprotein; glycoproteins are known to display unusual radiation sensitivity (13). Furthermore, high salt was required in order to keep avidin-PITC in solution and this led to complications in the analysis of the biotinylated enzyme. To overcome these difficulties, avidin was replaced with streptavidin which is similar in size, but lacks oligosaccharide moieties. The fluorescence tag was substituted with the enzyme peroxidase which eliminated the need for a high salt concentration. The biotin derivative was also changed from glucose-6-phosphate dehydrogenase to the fluorescent protein, B-phycoerythrin (bE).

Peroxidase is a single polypeptide glycoprotein of 44 kDa of which 34 kDa is protein (23). The target size observed here corresponds to the protein mass as found in all other glycoproteins (13). A small target size (14 kDa) for enzymatic activity of peroxidase (calculated from the data of Shikita and Hitano-Sato (24)) could not be confirmed.

The radiation sensitivity of peroxidase is not altered by coupling to streptavidin, which is a carbohydrate-free homotetramer (4 × 13.2 ≈ 53 kDa) (6, 7). These results indicate that energy deposited in streptavidin does not lead to inactivation of the peroxidase; damage to peroxidase occurs only by a primary ionization in the peroxidase moiety. Denaturing gel electrophoresis of SAPO revealed only two bands: peroxidase covalently bound to a streptavidin subunit and free streptavidin. HPLC analysis revealed that all the enzymatic activity eluted at the position of SAPO. These results demonstrate that in the covalently bound peroxidase, transfer of energy does not spread throughout the molecule. The energy transfer appears to be blocked at the thioether, 12-carbon linkage group between streptavidin and peroxidase. This energy blockage at a unique chemical structure, as in oligosaccharides (13), may help explain the scattered reports of radiation-resistant domains in other proteins (25).

The radiation inactivation of phycoerythrin gave a target of 29 kDa for fluorescence. boE is a fluorescent protein of M = 240,000 composed of 13 subunits (α + β). γ; α = 16 kDa; β = 17.5 kDa; γ = M; 40,000 kDa) with covalently attached tetrapyrroles (26). The target size appears to correlate with a dimer (α2, β2, or α + β). The presence of biotin and the biotinylated form of phycoerythrin showed the same target sizes of 29–31 kDa for fluorescence. As expected, the addition of a small molecule such as biotin (0.244 kDa) does not increase the radiation-sensitive mass.

It is likely that energy transfer from peroxidase into streptavidin (the opposite direction from that discussed above) also is blocked. In the SAPO-boE complex the target size for phycoerythrin fluorescence then would be expected to be independent of peroxidase. Rather, it would be the phycoerythrin target (30 kDa) with or without the streptavidin mass (subunit mass 13 kDa) depending on whether there were energy transfer across the noncovalent bonds. When the boE is coupled to SAPO, the radiation inactivation of fluorescence

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*Fig. 4. Loss of fluorescence in solutions of biotinylated phycoerythrin (boE) in the presence of transferrin irradiated frozen. Data points show average and S.D. from three independent experiments.*

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4 Subunit monomers of avidin and G6PDH from the completely dissociated bG6PDH−Av or TxR complexes were resolved on SDS-PAGE. In irradiated samples, the disappearance of these monomers led to target sizes equivalent to individual avidin and bG6PDH subunits. The latter result confirms the value obtained for the native G6PDH (22). These target sizes for loss of monomers corroborate the lack of major energy transfer beyond the polypeptide subunit.
does not change. The calculated target value is the same as those observed in the absence of the noncovalent interactions. This result indicates that energy deposited by radiation in either portion of the complex is not able to pass through these bonds, at least not in amounts sufficient to destroy the measured function.

**Interpretation**—The experiments with streptavidin-peroxidase and biotinylated phycoerythrin were performed both as an independent example as well as for the differences of these components from the parallel avidin-biotinylated G6PDH system. All of these experiments lead to the same conclusion: no evidence for major energy transfer across these bonds could be found in any of the biotin-avidin interactions.

There are two major objections to this interpretation: 1) both the enzymatic activity and fluorescence measurements could be due only to unbound molecules. A large excess of one of the components would yield a target size of just that molecule and thus the target size would be blind to the interacting molecules. Samples were examined which contained biotin to avidin in molar ratios from 1:1 to 9:1 which cover the entire range from excess biotinylated enzyme to excess avidin, yet the target sizes for both enzymatic activity and fluorescence did not change. A complication in interpretation arises since the activity of bG6PDH is reduced in the unbound molecules can be discounted. There are many interactions between avidin and streptavidin (28, 29). The ureido group forms hydrogen bonds involving five different amino acid residues on an avidin subunit, and another hydrogen bond forms at the valeryl carboxyl group. The tetrahydrothiophene portion of biotin forms hydrophobic interactions with four tryptophan residues, and there is a possible interaction between the biotin sulfor and a threonine hydroxyl group. According to Weber et al. (28), the ureido hydrogen bond interactions "predominate in stabilizing the biotin-protein complex."

A system analogous to the avidin-biotin interaction is found in double-stranded DNA, the DNA chains are joined only by hydrogen bonds. One of the direct effects of radiation in DNA is the scission of the phosphoribosyl backbone (a single strand break). The transfer of radiation energy across the hydrogen-bonded base pair (among other mechanisms) could lead to a nearby rupture of the opposite chain, resulting in appearance of a double-strand break. There is a very large body of literature showing that production of single-strand breaks (by high energy electrons, x- or y-rays) are more than an order of magnitude more common than double-strand breaks (30, 31). Thus significant energy transfer across these hydrogen bonds must be, at best, an infrequent event.

Since no energy transfer can be observed even between macromolecules joined by strong noncovalent interactions, the several reports of radiation damage from a single primary ionization appearing in different polypeptides of an oligomeric protein (5-11) are difficult to explain. The route and mechanism of energy transfer cannot be due to noncovalent bonds alone, no matter how strong their interaction. Other parameters must be considered, such as detailed conformational structure or subunit interactions which are unique to this population of proteins. Finally, the assumption that energy transfer occurs via a bond structure may itself be questioned.

**Acknowledgments**—We thank Drs. R. Kincaid, R. Horowitz, and D. Wright for their helpful suggestions.

**REFERENCES**


*On binding of biotin, a surface polypeptide loop of streptavidin moves so as to effectively bury the biotin in the protein interior, with only the carboxyl group accessible to the solvent (28, 29). Both the 5E and G6PDH were attached to this carboxyl group, and it has been shown that macromolecular binding at this position does not affect the binding to avidin (12). Nevertheless, it could be argued that these large molecules might prevent the surface loop from closing, and therefore the biotin interaction could be considerably weakened. Thus the noncovalent bonds in these examples might be so weak that the energy could not get across, and generalizations about strong noncovalent interactions would be unjustified. While the biotinylation of G6PDH is direct, 5E is attached to biotin through a 7-carbon spacer. It is likely that the streptavidin loop would be able to move, at least in the case of 5E, and maintain the strong interaction between biotin and streptavidin.*
Direct effects of radiation on the avidin-biotin system. Absence of energy transfer.
E S Kempner and J H Miller


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