*SUBCELLULAR PARTICULATE SYSTEMS AND THE PHOTOCHEMICAL APPARATUS OF RHODOSPIRILLUM RUBRUM**

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The photoactive pigments of the photosynthetic bacteria are believed to be localized in small spherical bodies called chromatophores.[1] Biochemical studies[2] have shown that chromatophore preparations catalyze a variety of light-dependent reactions (e.g., photophosphorylation of adenosine diphosphate) and they are, therefore, frequently considered to represent the functional equivalent of green plant chloroplasts. Electron micrographs[3] of thin sections of mature light-grown photosynthetic bacteria indicate that chromatophores are densely packed throughout the cell, and it has been known for some time that these bodies can be readily obtained from extracts of bacteria disrupted by sonic oscillation or mechanical abrasion. These observations have led to the impression that chromatophores are discrete morphological entities dispersed throughout the cytoplasm.

Sonic disruption of cells has been the most widely used method for preparing chromatophore suspensions. If sonication is extended beyond a critical time, however, photophosphorylation activity[4, 5] and the ability to produce molecular hydrogen photochemically[6] are lost. Continued sonic oscillation also leads to fragmentation of chromatophores.[7] In view of these results, it seemed likely that various properties of subcellular particles obtained by sonication might be significantly altered from those of the native photochemical system in the intact cell. Studies were therefore initiated to produce fragile cell-forms, such as protoplasts, of *Rhodospirillum rubrum*, from which the photochemical particulate system could be released by mild procedures.[8] These preliminary experiments disclosed that virtually all of the pigmented material in "lysates" (made by osmotic shock) of *Rhodospirillum* protoplasts was readily sedimented at low centrifugal force. Since free chromatophores obtained by the usual procedures are sedimented only at much higher forces (e.g., 46,000 to 54,000 × g for 50 minutes[2]) it was suggested that (*in vivo*) these bodies are associated with membranous components of the cell.

The present experiments with several types of altered cell-forms of *Rhodospirillum* offer further evidence that the system responsible for photochemical generation of adenosine triphosphate is normally associated with the cytoplasmic membrane or a system of membranous extensions penetrating the cytoplasm.

Materials and Methods. *Rhodospirillum rubrum* (strain S1) was grown photosynthetically at approximately 30°C in either the G3X or G5 media described by Kohlmiller and Gest.[9] Both media contain L-glutamic and DL-malic acids as major components, but differ with respect to their content of complex supplements (yeast extract, peptone).

Phase contrast examinations were made with the Leitz Ortholux microscope.

Rates of photophosphorylation of adenosine diphosphate (ADP) were estimated by determining utilization of inorganic phosphate as outlined by Frenkel.[4] Bacteriochlorophyll estimations were made using the methods of Cohen-Bazire *et al.*[10]

Protein concentrations (Fig. 1) were determined by the Folin-phenol method of Lowry *et al.*[11] on samples precipitated with trichloroacetic acid (crystalline bovine serum albumin used for standards).

Special reagents were purchased from the following sources: polymixin B sulfate, Nutritional Biochemicals Corp. (Cleveland, Ohio); phenazine methosulfate (PMS) and tris (hydroxymethyl) aminomethane (Tris), Sigma Chemical Co. (St Louis, Missouri); ADP, Sigma Chemical Co. and Pabst Laboratories (Milwaukee, Wisconsin); lysozyme, Sigma Chemical Co. and Armour Laboratories (Chicago, Illinois).

Results.—Protoplasts and "ghosts": Typical spiral-shaped cells of *R. rubrum* can be converted to nonmotile spherical protoplasts by procedures effective with a variety of other bacteria, e.g., by addition of penicillin (approx. 1000 units/ml) and high concentrations of an osmotic stabilizer such as sucrose to cultures actively growing in the light or by exposure of resting cells to lysozyme in the presence of versene (ethylenediaminetetraacetic acid) and sucrose. The lysozyme-versene method is technically more suitable for obtaining the quantities of *R. rubrum* protoplasts required for metabolic experiments and is described in detail in the preliminary communication of Karunairatnam, Spizizen, and Gest.[8] The latter investigators also compared several metabolic activities in intact cell and protoplast suspensions and observed qualitative similarity, except for the inability of protoplasts to photoevolve H_2. Absence of this activity may have been due to leakage of essential cofactors from the protoplasts, which very likely have an altered permeability as compared with whole cells.

R. rubrum lysozyme-protoplasts resemble those of other bacteria in many respects. Thus, dilution of concentrated suspensions (in 0.6 *M* sucrose) with water or addition of water to packed protoplasts usually yields gelatinous or stringy preparations. Considerable variability has been noted with different batches of protoplasts in this regard. In some instances, gelatinous preparations have been encountered during the final stages of protoplast preparation (with sucrose present). On the other hand, with other batches, the first treatment with water may yield "flaky" suspensions, and gels are sometimes observed only after several washes. These and numerous other observations suggest that *R. rubrum* protoplasts may not be as osmotically-fragile as the corresponding forms of Gram positive bacteria. The viscosity or "flakiness" observed in preparations made hypotonic by dilution with water is presumably due to release of desoxyribonucleic acid since it can be diminished or eliminated by addition of desoxyribonuclease.

Microscopic examination under phase contrast discloses that *R. rubrum* protoplasts enlarge upon dilution of sucrose suspensions with water and are converted to pale "ghosts" which usually appear to contain a number of opaque granules. These forms are strikingly similar in appearance to the "ghosts" derived from osmotically-shocked protoplasts of *Bacillus megaterium*.[12] Centrifugation of *R. rubrum* "ghost" suspensions at relatively low speeds (e.g., 14,500 × g for 20 minutes) results in a dark red-colored sediment and a pale pink supernatant liquid. Subsequent washing of the pellet with water frequently releases a small fraction of the pigmented material in a form not as readily sedimentable, but the bulk of the pigment remains associated with the "ghost" structures. The pigmented "ghosts" contain the functional photochemical apparatus which catalyzes photophosphoryla-

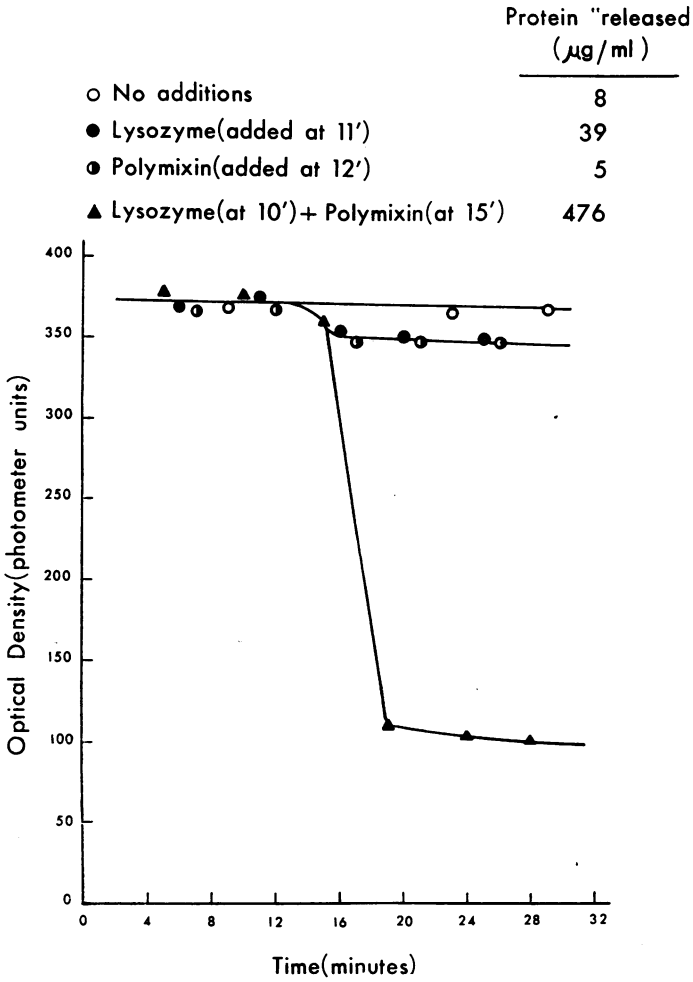

FIG. 1.—"Lytic" effect of sequential lysozyme-polymixin treatment on intact cells of *Rhodospirillum rubrum*. The cells (30 hours old) were grown photosynthetically in G5 medium, washed twice with 0.02 *M* Tris pH 8, and resuspended in 0.05 *M* Tris pH 8. 5.0-ml. aliquots (containing 9 mg dry weight of cells) of suspension were supplemented with 0.8 (▲), 0.9 (●, ◑), or 1.0 ml of water (○). Where indicated, 0.1-ml amounts of lysozyme (6 mg/ml) or polymixin B (2.5 mg/ml) solutions were added and the suspensions rapidly mixed. Optical densities were measured with the Klett-Summerson photoelectric colorimeter (No. 66 filter). Incubation temperature, 37°C. At 30 minutes, the suspensions were cooled, centrifuged to remove pigmented material, and the supernatant fluids analyzed for protein. The value given for protein "released" in curve ▲ may include some contribution, presumably 39 μg/ml or less, from unadsorbed lysozyme.

tion of adenosine diphosphate (Table 1).

The foregoing and similar experiments not detailed here indicate that photophosphorylation activity in "ghost" (or "lysed protoplast") suspensions shows many over-all similarities with the process as it occurs in preparations of free chromato-

TABLE 1

PHOTOPHOSPHORYLATION OF ADENOSINE DIPHOSPHATE BY "GHOSTS" OF *R. RUBRUM*

Vessel	"Cofactor" Additions*	Atmosphere	μmoles Inorganic Phosphate Esterified per Hour per mg Bacterio-chlorophyll
1	None	Helium	0
2	None	Hydrogen	0
3	Mg^{++} (20 μmoles) + succinate (0.5 μmole)	Helium	16.9
4	Mg^{++} + succinate	Hydrogen	15.5
5	Mg^{++} + succinate + phenazine methosulfate (25 μg/ml)	Helium	57.7

* *Incubation conditions:* Warburg vessels of approximately 10 ml capacity, containing a final fluid volume of 2 ml were employed. Each vessel contained 0.8 ml of "ghost" suspension, 400 μmoles glycylglycine pH 7.6, 15 μmoles inorganic phosphate, and 10 μmoles adenosine diphosphate. Other additions as listed above. Temperature, 30°C. Light intensity, ~1000 foot-candles.

"Ghost" preparation: Light-grown cells (40 hours old, from G3X medium) were converted to protoplasts by the ysozyme-versene method. 10 ml of protoplast suspension (in 0.6 *M* sucrose; derived from the cells in 280 ml of medium) were centrifuged at 14,500 × g for 20 min and the pellet resuspended in 10 ml. of water. 0.6 mg of Worthington desoxyribonuclease was added to the "flaky" suspension and after incubation at 30°C for 5 min, the suspension was recentrifuged. The resulting pellet was washed once more with water, resuspended in 10 ml of water, and uniformly dispersed using a hand-operated Ten Broeck homogenizer.

phores.[4, 5] Thus, with both types of preparations the reaction is light-dependent, requires the presence of an acceptor such as adenosine diphosphate, is stimulated by catalytic amounts of phenazine methosulfate (provided succinate is present at low concentration) and is not significantly influenced by the presence of a hydrogen atmosphere. Mg^{++} and small amounts of succinate, or other reducing agents, are required for optimal photophosphorylation by purified free chromatophores but not by crude preparations obtained by sonic oscillation.[4] From the results of Table 1, it appears that these or similar "cofactors" must be lost during preparation of the washed "ghosts."

Protoplasts and "ghosts" of *R. rubrum* are relatively fragile structures and can be disrupted in various ways with the apparent release of chromatophores, e.g., by shaking with Ballotini beads in the Mickle apparatus or by exposure to surface active agents such as sodium lauryl sulfate. Salton[13] has noted that isolated cell wall preparations of *R. rubrum* and *E. coli* appear to be dissolved by sodium lauryl sulfate and similar anionic detergents. The possible usefulness of sodium lauryl sulfate as a dispersing agent for *Rhodospirillum* altered cell-forms is, however, somewhat limited by the fact that the detergent interacts, more or less rapidly depending on its concentration, with the pigment system causing marked visible color changes (the 880 mμ absorption peak of bacteriochlorophyll may also be significantly depressed).

Effects of polymixin: The structure of the outer integument of typical Gram negative bacteria appears to be considerably more complex than the cell walls of Gram positive organisms such as the bacilli.[14] One of the outstanding differences between the two types is the presence of a high proportion of lipid or lipoprotein in the surface structures of Gram negative cells. The studies of B. A. Newton,[15] Few,[16] and others indicate that the cyclic polypeptide antibiotic polymixin (polymyxin) reacts with lipid components of cytoplasmic membranes of sensitive Gram negative bacteria, causing disorganization of the membrane and an attendant loss of specific permeability properties. It might be expected that polymixin would also affect lipoprotein wall components. These considerations prompted tests with this

antibiotic and it was found that exposure of resting cells of *Rhodospirillum*, in 0.05 *M* Tris buffer pH 8, to 250 μg/ml of polymixin B resulted in the formation of granulated protoplast-like bodies over the course of several hours.

Since polymixin can induce the formation of protoplast-like bodies and other aberrant morphological forms from spiral-shaped cells, it seems likely that lipid components of the wall mosaic must play a significant role in maintaining the structural rigidity of the cell. This conclusion is also supported by the facts that cells of *R. rubrum* growing in the presence of 60 μg polymixin B/ml (in G3X medium) are bacilli-form, rather than spiral-shaped, and that treatment of normal spirals with 10 per cent phenol destroys the characteristic shape yielding rod-like morphological elements.

"Circle" preparations: Protoplasts or protoplast-like bodies produced from *R. rubrum* are ordinarily quite opaque as viewed under phase contrast. In numerous types of preparations, e.g., of intact cells frozen and thawed in the presence of lysozyme and in "ghost" suspensions treated with sodium lauryl sulfate, we have observed spherical bodies which appear to be completely transparent and sharply delineated by a thin membrane; an adhering patch of phase-dense material is sometimes revealed by careful focusing. These forms closely resemble the empty "ghosts" which Weibull[17] prepared by treatment of the Gram positive *Bacillus megaterium* with lysozyme in the absence of an osmotic stabilizer. Because of their microscopic appearance we use the operational term "circle" to represent such structures.

"Circles" can be rapidly made in almost 100 per cent yield by briefly exposing spiral-shaped cells to lysozyme and then to polymixin B under the conditions given for Figure 1. With lysozyme alone, the spiral morphology is unaffected but a small number of protoplasts are invariably observed in the suspensions.[18] Polymixin alone under these conditions, does not cause observable morphological alterations.

When a suspension of normal spirals is converted to "circles" by the synergistic action of lysozyme and polymixin B, the optical density decreases rapidly upon addition of the antibiotic and is attended by an extensive release of protein (see Fig. 1). The suspension clarifies and superficially appears to have lysed completely. Control suspensions with no supplements, or with lysozyme or polymixin added individually, do not show significant decreases in optical density or liberation of protein.

The "lysis" of *R. rubrum* by sequential lysozyme-polymixin treatment is greatly retarded if the order of addition is reversed. This effect is reminiscent of Repaske's observations[19] that, although the addition of versene and Tris stimulated rapid lysis by lysozyme of *Escherichia coli*, *Azotobacter vinelandii*, and several pseudomonads, in the case of *E. coli* at least, preincubation with versene before lysozyme addition markedly reduced the rate of lysis. The interpretation was nevertheless offered that versene exerts its effect by chelating a metal on the cell surface, thereby exposing the "lysozyme-substrate." Warren et al.[20] similarly have suggested that polymixin causes exposure of "lysozyme-substrate," since in their studies on the combined action of lysozyme and polymixin on several Gram negative bacteria, increased susceptibility to lysis was achieved by pretreatment with polymixin. It would be of interest, in view of the present findings with *R.*

rubrum, to determine whether the organisms examined by Warren et al. are lysed even more rapidly by preexposure to lysozyme before polymixin addition. Perhaps these organisms would also be converted to "circles" by such treatment.

The experimental results with *R. rubrum* appear to lend themselves to the interpretation that the "lysozyme-substrate" in the cell surface of this organism is readily accessible, but is involved together with polymixin-sensitive components (presumably lipid or lipoprotein) in conferring the characteristic shape to the intact normal cell. It is conceivable that versene, like polymixin, interacts with lipid constituents of the cell "envelope," and that, in fact, prior disruption of these elements may somehow retard the action of lysozyme.

Practically all of the pigmented material in "circle" suspensions of *R. rubrum* is sedimented by centrifugation at 6,300 × g for 15 minutes. In contrast with intact cells and protoplasts, the pigmented "circle" pellet is translucent and is similar in this respect to pellets of free chromatophores. It may be noted that "circles" do not contain any of the large opaque granules characteristic of *R. rubrum*, which argues against the possibility of physical entrapment of free chromatophores by the limiting (damaged) membrane. "Circle" preparations catalyze the photophosphorylation of adenosine diphosphate as illustrated by the data of Table 2.

TABLE 2

PHOTOPHOSPHORYLATION OF ADENOSINE DIPHOSPHATE BY "CIRCLES" PREPARED FROM *R. RUBRUM*

Tube	Incubated in	"Cofactor" Additions*	Δ μmoles Inorganic Phosphate per 40 Minutes†
1	Light	None	0
2	"	Mg^{++} (20 μmoles)	−0.3
3	"	Succinate (0.5 μmole)	−0.4
4	"	Phenazine methosulfate (25 μg/ml)	−1.5
5	"	Mg^{++} + succinate	−0.4
6	"	Mg^{++} + succinate + PMS	−4.3
7	Dark	Mg^{++} + succinate + PMS	+0.2

* *Incubation conditions:* As in Table 1, except that the suspensions were incubated in square Thunberg tubes, open to the atmosphere. Each tube contained 0.5 ml of "circle" preparation, made as described below.

† Change from zero time control.

"Circle" preparation: Light-grown cells (24 hours old) derived from 500 ml of G5 medium were converted to "circles" under the conditions given for Figure 1 (▲), but at a greatly increased scale (750 ml starting volume). A considerable amount of floating, pigmented material was removed from the surface of the "lysed" suspension by careful siphoning and the remainder then centrifuged at 1,460 × g for 10 min to remove stringy pigmented masses. "Circles" were collected from the supernatant fluid by centrifugation at 7,000 × g for 15 min. The resulting pellet was washed once with 20 ml of 0.05 *M* glycylglycine buffer pH 7.6 (containing 1.9 μmoles inorganic phosphate per ml), resuspended in 7 ml of the same buffer, and homogenized with a hand-operated Ten Broeck.

With all three "cofactors" (Mg^{++}, succinate, and PMS) present, the rate of photophosphorylation shown by this particular preparation was 77 μmoles inorganic phosphate esterified per hour per mg bacteriochlorophyll. In the absence of the dye, however, "circle" preparations show unexpectedly low activity. Petrack[21] has recently reported that cell-free extracts of blue-green algae show a similar dependence on PMS for active photophosphorylation.

At the present time it is not known whether the photochemically active "circles" produced from *R. rubrum* by the combined action of lysozyme and polymixin represent cytoplasmic membranes, membranes with adhering surface components, or several layers of the integument which are considerably disorganized but still fused together. Analysis of a circle preparation for hexosamine,[22] a characteristic cell surface constituent, revealed its presence to the extent of approximately 80 per cent of the amount found in normal cells (~400 μg, calculated as glucosamine, per

100 mg dry weight of cells). This finding is of interest but, considering our inadequate knowledge of the structure of the Gram negative cell integument, does not permit an unambiguous choice among the several possibilities.

Treatment of typical "circle" suspensions with sodium lauryl sulfate causes a decrease in optical density (No. 66 filter), resulting from disintegration with the release of pigmented fragments. For example, in an experiment with 1 mg detergent/ml, a negligible pellet was obtained by centrifugation at 26,000 × g for 30 minutes; the bacteriochlorophyll in the supernatant fluid was markedly affected, however (ratio of optical density at 880 mμ to optical density at 800 mμ ≅ 1.3). With 0.5 mg/ml, a thin translucent pellet was observed and the pink supernatant fluid showed a corresponding optical density ratio of approximately 6, which is characteristic for bacteriochlorophyll in chromatophores of *R. rubrum*.[1] It is possible that under proper circumstances sodium lauryl sulfate, or similar surface active agents, may release active "sub-units" from the supporting membranous structures.

Discussion.—Many investigators have noted that an appreciable fraction of the photosynthetic pigments in biochemically active cell-free extracts of purple bacteria is sedimentable at relatively low centrifugal force. Ordinarily, this material is discarded and free chromatophores are isolated (by high speed centrifugation) from the supernatant fluids resulting from centrifugation at 25,000 × g for 30 minutes or longer. The present experiments demonstrate that mild lytic procedures, such as osmotic shock of protoplasts, do not release chromatophores in significant quantity, but rather that the functional photophosphorylation apparatus remains associated with large membranous structures which are sedimentable at low speeds. It is therefore suggested that the photoactive pigment system of the photosynthetic bacteria is associated with the (lipoprotein) cytoplasmic membrane and/or a reticulum of membranous extensions penetrating the cytoplasm. A corollary of this interpretation is that the bacterial pigment complex does not normally exist in the form of independent cytoplasmic inclusions comparable to the chloroplasts of higher plants.

The foregoing conception would reconcile our findings with a number of observations which until now seemed contradictory. Studies[3] of thin sections of light-grown photosynthetic bacteria by electron micrography indicate that chromatophores, in cells grown in the usual manner, are densely packed throughout the cytoplasm. On the other hand, the results of immunochemical (and other[7]) studies with *Chromatium* by J. W. Newton[23] have led him to conclude that "the photosynthetic apparatus of purple bacteria is not necessarily a unique, discrete intracellular entity, since it contains macromolecular configurations common to the cell surface on one hand and certain 'intracellular' proteins on the other. It seems advisable, therefore, provisionally to consider it a part of a more complex organizational state within the cell." It is pertinent to note that "cell wall" preparations obtained from *R. rubrum* by the usual technique (disruption in the Mickle shaker) show only slight pigmentation. The presence of a small amount of pigment could be explained as being due to contamination of the outer cell-envelope by adhering inner membrane structures. Few[24] has, in fact, reported that prolonged shaking of Gram negative bacteria in the Mickle disintegrator is required to produce cell walls devoid of the "secondary inner layer."

The mechanism and dynamics of chromatophore formation are still obscure. It is of special interest in this connection that Frenkel[25] has recently observed that *very young* light-grown cells of *R. rubrum* do not contain chromatophores, but instead contain smaller (200–300 Å) photochemically active particles. Furthermore, similar particles are obtained if older cells grown in the usual way are sonicated in viscous media.[2, 25] These pigmented particles are reported to be as photochemically active as chromatophores. It is tempting to speculate that the small "photosynthetic sub-units" are derived from the cytoplasmic or similar membranes. Cytoplasmic membranes of bacteria are fragile structures, known to be readily disintegrated by sonic oscillation and other vigorous disruption procedures[26, 27] and, accordingly, it has been suggested that various subcellular particulate species observed in bacterial extracts made by these techniques represent fragments of such membranes. This view is supported by increasing evidence that many "particle-bound" enzyme systems can also be shown to be integral parts of cytoplasmic membranes[26, 28] or the cell "envelope."[29]

Summary.—Osmotic lysis of altered fragile cell-forms (protoplasts) of the Gram negative photosynthetic bacterium *Rhodospirillum rubrum* does not release significant amounts of free chromatophores, but results in the formation of pigmented membranous structures ("ghosts") which are capable of photophosphorylating adenosine diphosphate. A second type of photochemically active form, which closely resembles the "empty ghosts" of certain Gram positive bacteria, can be prepared by the sequential action of lysozyme and the polypeptide antibiotic polymixin B on resting cells. Both types of preparations, in contrast with free chromatophores, are readily sedimented at low centrifugal force. On the basis of the current findings and other observations from the literature, it is concluded that *in vivo* the photochemical apparatus of the photosynthetic bacteria is associated with the cytoplasmic membrane and/or membranous extensions in the cytoplasm of the cell.

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*A CRITERION FOR ORBITAL HYBRIDIZATION AND CHARGE DISTRIBUTION IN CHEMICAL BONDS**

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Although parameters related to orbital hybridization and charge distribution have long been used in descriptions of the chemical bond,[1] reliable values for these quantities have been very difficult to obtain. In this report, we suggest that electron-coupled nuclear spin interactions, when interpreted in terms of the theory of localized electron pairs, can serve to measure the extent of orbital hybridization and bond polarization.

The coupling constant $A_{NN'}$ (in units of cps) for a pair of nuclei with spins $\mathbf{I}_N$ and $\mathbf{I}_{N'}$ is defined by the expression

$$E_{NN'} = hA_{NN'}\,\mathbf{I}_N \cdot \mathbf{I}_{N'} \qquad (1)$$

where $E_{NN'}$ is the interaction energy, which can be determined by an analysis of the