

## STUDIES ON ANTIGENICITY

### THE RELATIONSHIP BETWEEN IN VIVO AND IN VITRO ENZYMIC DEGRADABILITY OF HAPTEN-POLYLYSINE CONJUGATES AND THEIR ANTIGENICITIES IN GUINEA PIGS\*

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Hapten conjugates of synthetic poly amino acids are useful tools for the investigation of the metabolism of antigen, which is an early step in the immune response. Previous studies have shown that approximately 25 per cent of random-bred guinea pigs and 100 per cent of the highly inbred strain 2 guinea pigs are capable of developing an immune response to lightly coupled hapten-poly-L-lysine (PLL) conjugates (1-5). These animals have been termed "responders," whereas guinea pigs incapable of responding immunologically to hapten-PLL conjugates were termed "non-responders." The inability of non-responder guinea pigs to recognize hapten-PLL conjugates as antigen is not due to an absence of antigenic determinants in these conjugates, since these guinea pigs can synthesize antihapten antibodies when immunized with protein conjugates of these same haptenic determinants.

Individual random-bred guinea pigs immunized with PLL<sup>1</sup> conjugates of 4 structurally unrelated haptens either show immune responses against all 4 haptens or against none (2). In addition, the ability to develop an immune response to hapten-PLL conjugates was shown to be transmitted genetically as a unigenic, autosomal Mendelian dominant trait (3). These findings suggested that the difference between responder and non-responder guinea pigs may reside in their abilities to metabolize properly the PLL carrier. The genetic findings indicate further that the differences between responder and non-responder guinea pigs may be the presence, in responders, of a single essential metabolic enzyme, either an enzyme responsible for degradation of the PLL carrier (e.g. a cathepsin) or an enzyme involved in a subsequent metabolic operation on the PLL carrier, which leads to the formation of the specific inducer bearing the antigenic determinant.

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<sup>1</sup> Abbreviations: BPO, benzylpenicilloyl; DNP, 2,4-dinitrophenyl; H<sup>3</sup>DNP, tritium-tagged DNP; Fluor, fluorescein haptenic group. PLL, poly-L-lysine; PLL-S, exhaustively succinylated PLL; PDL, poly-D-lysine. Subscripts refer to average numbers of lysine residues per polypeptide chain. PBS, phosphate-buffered saline (0.15 M NaCl, 0.02 M phosphate, pH 8.0); TBS, tris-buffered saline (0.15 M NaCl, 0.02 M tris-(hydroxymethyl)aminomethane, pH 8.0).

Contrasting with the antigenicity of hapten-PLL conjugates, benzylpenicilloyl (BPO)-PLL conjugates whose free amino groups have been exhaustively acylated with succinic anhydride (4), and lightly coupled BPO-poly-D-lysine (PDL) conjugates (5, 6) are not immunogenic, even in responder animals.

Enzymatic degradation of macromolecular antigens as an initial step in the immune response has been postulated to explain the non-antigenicity of materials such as copolymers of poly-D-amino acids (7), hapten conjugates of poly-D-amino acids (5, 6, 8), polyvinyl pyrrolidone (PVP) (9), and alkali racemized proteins (10), which are presumed to be undegradable by catheptic enzymes of the reticuloendothelial system.

The present work investigates whether the immunogenicity of hapten-PLL conjugates, exhaustively succinylated hapten-PLL conjugates, and hapten-PDL conjugates is related to their abilities to be degraded enzymatically by immunological tissues. Enzymatic degradation of these 3 kinds of hapten-polylysine conjugates by aqueous extracts of spleens from responder and non-responder guinea pigs has been investigated. In addition, *in vivo* studies were carried out which measured the urinary excretion of low molecular weight degradation products of these conjugates after their intraperitoneal injection. It was found that both responder and non-responder guinea pigs can degrade succinylated and non-succinylated PLL conjugates into small molecular fragments, but they cannot degrade poly-D-lysine conjugates. These studies demonstrate that in addition to the known requirements for antigenicity of macromolecules, *i.e.* the presence of antigenic determinants and the ability to be degraded by immunological tissues, the resulting degradation products must undergo certain additional, as yet unidentified, specific metabolic steps in order to induce an immune response.

#### *Materials and Methods*

*Conjugates.*—Poly-L-lysine<sub>525</sub> HBR (Lot 22), poly-L-lysine<sub>286</sub> HBR (Lot 28), and poly-D-lysine<sub>144</sub> (Lot DL-10) were purchased from Pilot Laboratories, Waltham, Massachusetts. The average degrees of polymerization of 525, 286, and 144 lysine units respectively were calculated from intrinsic viscosity measurements (manufacturer's analyses). Crystalline fluorescein isothiocyanate (isomer I, Lot 307649) was purchased from Baltimore Biological Laboratories, Baltimore. H<sup>3</sup>-dinitrochlorobenzene (specific activity of 25 mc/mole) was purchased from New England Nuclear Corporation, Boston. Fluorescein-polylysine conjugates (Fluor-PLL<sub>525</sub>, and Fluor-PDL<sub>144</sub>) were prepared by reaction of the polylysine preparations with 0.12 molar equivalents (per mole of lysine  $\epsilon$ -NH<sub>2</sub>) of fluorescein isothiocyanate in aqueous solution in a pH stat, 25°C, for 90 minutes with the pH maintained between 8.8 and 9.0 by additions of 1 N NaOH. The reaction mixture was freed from low molecular weight side reaction products by 4 dialyses against 2 M NaCl followed by 3 dialyses against water. An aliquot of the Fluor-PLL<sub>525</sub> conjugate was exhaustively succinylated by acylation with 3 molar equivalents (per mole of  $\epsilon$ -NH<sub>2</sub>) of succinic anhydride in aqueous solution in a pH stat, once at pH 11.7 and again at pH 9.5–10. The succinylated conjugate, Fluor-PLL<sub>525</sub>-S, was purified by repeated dialyses against water. H<sup>3</sup>DNP-PLL<sub>286</sub> and H<sup>3</sup>DNP-PDL<sub>144</sub> were prepared by reaction of 0.7 per cent solutions of polylysine base in 0.25 M carbonate buffer (pH 11.5) with

0.05 and 0.3 molar equivalents (per mole  $\epsilon$ -NH<sub>2</sub>) of H<sup>3</sup>-dinitrochlorobenzene in dioxane. The final concentration of dioxane in the reaction solution was 15 per cent v/v. The reaction solution was stirred at room temperature for 24 hours. The conjugates were freed from low molecular weight radioactive side reaction products by 2 dialyses against 0.5 liter volume of 0.01 M dinitrophenol (pH 8.0), 3 dialyses against 1 M NaCl, and 6 dialyses against 0.15 M NaCl. An aliquot of H<sup>3</sup>DNP-PLL<sub>286</sub> was exhaustively succinylated by the method given above. Conjugate solutions were assayed for polylysine concentrations by duplicate microKjeldahls (11), and for DNP concentrations by spectrophotometry at 360 m $\mu$ . Average numbers of DNP haptenic groups per mole conjugate were 5 for H<sup>3</sup>DNP<sub>5</sub>-PLL<sub>286</sub> and H<sup>3</sup>DNP<sub>5</sub>-PLL<sub>286</sub>-S, and 30 for H<sup>3</sup>DNP<sub>30</sub>-PDL<sub>144</sub>.

*Spleen Extracts.*—Spleens from responder (strain 2) guinea pigs and non-responder random-bred guinea pigs were dissected free of connective tissue, immediately frozen in dry ice powder, and stored frozen at  $-20^{\circ}\text{C}$  for 1 to 4 weeks before use. Extracts of responder and non-responder spleens were prepared from groups of 6 spleens weighing about 5 gm by methods similar to those described by Press *et al.* (12). Spleens were thawed, minced, suspended in 20 ml of PBS, and homogenized for 30 minutes in a Virtis "45" homogenizer, Virtis Co., Gardiner, New York. Solid material was centrifuged off at 12,000 rpm and extracted once with 10 ml of PBS. The extract was adjusted to pH 4.9 with 0.1 N HCl, and the tan precipitate which formed was centrifuged off. The resulting light brown opalescent spleen extract (final volume, 25 ml) was stored frozen at  $-20^{\circ}\text{C}$  for 1 week before use.

*Enzymatic Degradation of Conjugates.*—Methods similar to those summarized in references 12 and 13 were used. The Fluor-PLL<sub>525</sub>, Fluor-PLL<sub>525</sub>-S, and Fluor-PDL<sub>144</sub> conjugates were diluted to concentrations of 2.0 mg/ml in 0.15 M NaCl. Incubation mixtures were made up by mixing 1.0 ml of spleen extract, 0.1 ml 1 M acetate buffer, pH 4.9, and 0.1 ml of 1 M cysteine HCL with 1.0 ml of the conjugate solutions. Control mixtures were identical except 1.0 ml of 0.15 M NaCl was substituted for the conjugate solution. Final pH of all mixtures was  $4.8 \pm 0.1$ . All mixtures were incubated at  $37^{\circ}\text{C}$  for 20 hours. After incubation, the Fluor-PLL<sub>525</sub>-S mixture showed a moderate amount of precipitation; the others were only slightly turbid.

*Analysis of the Digestion Mixtures.*—Descending paper chromatography was carried out on No. 1 Whatman paper using 80 per cent ethanol as the developing solvent. Vertical starch gel electrophoresis was carried out by the method of Smithies (14) at pH 8.5, 8 v per cm, for 6 hours at room temperature. Chromatograms and starch gels were examined under "long-wave" fluorescent light for fluorescent spots.

*Guinea Pigs.*—Random-bred and strain 2 guinea pigs were immunized with 100  $\mu\text{g}$  of benzylpenicilloyl<sub>24</sub>-PLL<sub>316</sub> in complete Freund's adjuvant as described previously (2), and tested for immune responses by skin test with 10  $\mu\text{g}$  of the immunizing conjugate on the 14th day, and again on the 21st day. All 5 strain 2 animals showed strongly positive Arthus and delayed skin reactions (responders). Random-bred guinea pigs were divided into groups of responders and non-responders according to the immune response to this immunizing conjugate, as described previously (2, 3).

*In Vivo Metabolic Studies.*—Responder and non-responder guinea pigs were injected intraperitoneally with the H<sup>3</sup>DNP-polylysine conjugates to be studied. The animals were placed in individual metabolic cages, and daily urines were collected. After measuring the daily urinary output, 0.1 ml samples were mixed with 15 ml of the following solution: naphthalene, 60 gm; PPO (2,5-diphenyloxazole), 4 gm (Packard Instrument Company, New York); dimethyl-POPOP (1,4-bis-2,4-methyl-5-phenyloxazolyl)-benzene, 0.2 gm, (Packard Instrument Company); methanol, 100 ml, ethylene glycol, 20 ml and dioxane to make 1000 ml. The radioactivity was counted in a scintillation counter. The amount of radioactivity excreted daily was calculated and recorded as percentage of the radioactivity injected.

## RESULTS

*Degradation of Fluorescein-Polylysine Conjugates by Spleen Extracts from Responder and Non-Responder Guinea Pigs.—*

Aqueous extracts of spleens removed from responder (strain 2) and non-responder guinea pigs were incubated with fluorescein conjugates of PLL, succinylated PLL, and PDL. Incubation was carried out at pH 4.8 at 37°C for 20 hours. Enzymatic degradation of the polylysine carriers could be detected by analyzing the digestion mixtures for the appearance of new, low molecular weight, fluorescein peptides by paper chromatography and by vertical starch gel electrophoresis.

Fig. 1 shows a drawing of typical paper chromatogram of incubated mixtures of non-responder spleen extract with the 3 conjugates. Degradation of the poly-L-lysine and of the succinylated PLL conjugates is demonstrated by the appearance of several new fluorescent spots in the chromatogram. In contrast, no new fluorescent spots appeared in the chromatogram of the incubated digestion mixture of the PDL conjugate. The faint fluorescent spot present in the chromatogram of the undigested PDL conjugate is probably a trace of hydrolyzed fluorescein isothiocyanate present as an impurity in this conjugate. Controls consisted of identical mixtures of spleen extract and fluorescein conjugates, which had not been incubated, but were chromatographed immediately after mixing. They showed no fluorescent spots other than those at the origin. Similar experiments were carried out with responder (strain 2) guinea pig extracts with identical results.

The incubated and non-incubated (control) digestion mixtures were analyzed also by vertical starch gel electrophoresis at pH 8.5. A typical run is illustrated in Fig. 2. Identical results were obtained for spleen extracts from responder and non-responder guinea pig spleens. Enzymatic degradation of the poly-L-lysine and the succinylated PLL carriers is indicated by the appearance of two new fluorescent spots which migrated toward the anode. By this method also there was no evidence of enzymatic degradation of the fluorescein-PDL conjugate by the spleen extracts.

*In Vivo Degradation of Hapten-Polylysine Conjugates by Responder and Non-Responder Guinea Pigs.—*

The degradation *in vivo* by responder and by non-responder guinea pigs of DNP-polylysine conjugates was studied by measuring the urinary excretions of radioactivity following the intraperitoneal injection of H<sup>3</sup>-labeled DNP conjugates of PLL, of succinylated PLL, and of PDL. In the first experiments, 4 responders and 4 non-responders were injected intraperitoneally with 4.3 mg of H<sup>3</sup>DNP-PLL and their urines were collected daily.

Fig. 3 shows the urinary excretions of radioactivity for 3 days following the injection of H<sup>3</sup>DNP-PLL expressed as percentage of radioactivity injected. The responder and the non-responder guinea pigs excreted in their urines approximately equal quantities of radioactive degradation products



of  $H^3$ DNP-PLL. Daily urines were collected for a total of 8 days from 2 responders and from 3 non-responders injected with  $H^3$ DNP-PLL. The 3 non-responders excreted totals of 56, 70, and 80 per cent of the injected radioactivity, and the 2 responders excreted totals of 70 and 90 per cent. The radioactive degradation products of  $H^3$ DNP-PLL found in the urine are of comparatively small molecular sizes as demonstrated by the following: (a) when

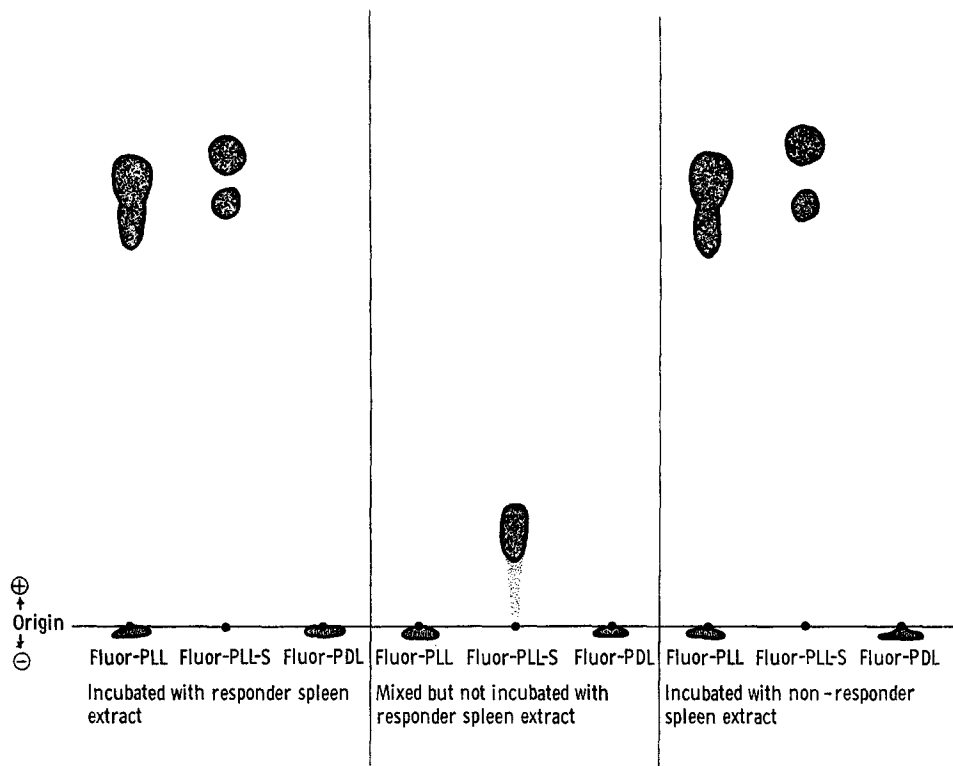


FIG. 2. Drawing of vertical starch gel electrophoresis (pH 8.5) of incubated mixtures of fluorescein-polylysine conjugates with aqueous extracts of responder and non-responder guinea pig spleens. Dark spots are areas of fluorescence seen under ultraviolet light.

dialyzed against 0.15 M saline, the concentrations of radioactivity inside and outside the dialysis bag were the same; (b) on sephadex G-25 gel filtration, the urinary radioactivity was eluted with the inside volume. In this system, undegraded  $H^3$ DNP-PLL was eluted with the outside volume, whereas  $\epsilon$ -DNP-lysine was eluted with the inside volume.

In the second experiments, 2 non-responder guinea pigs were injected intraperitoneally with 2.0 mg of  $H^3$ DNP-PLL (group 1), a second group of 2 non-responders with 2.0 mg of

succinylated  $H^3$ DNP-PLL, and a third group of 2 non-responders and 2 responders with 1.4 mg of  $H^3$ -DNP-PDL. The daily urinary excretions of radioactivity were followed.

Fig. 4 shows the average percentages of injected radioactivity excreted by each group of guinea pigs. The succinylated PLL conjugate was degraded at least as rapidly as was the non-succinylated PLL conjugate. Similar to the results obtained in the *in vitro* studies, no evidence of degradation of  $H^3$ DNP-PDL was obtained. In each of the 4 guinea pigs injected with  $H^3$ DNP-PDL, the total urinary excretion of radioactivity over a 5 day period was less than 1 per cent of the injected dose.

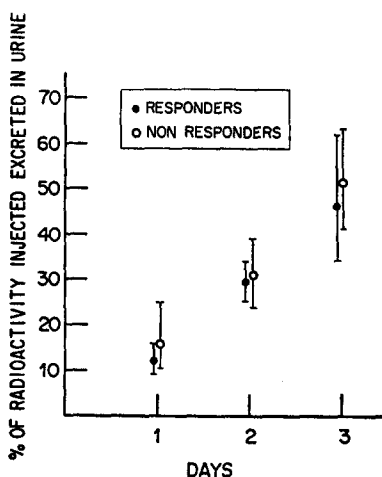


FIG. 3. Cumulative urinary excretions of radioactivity from responder and non-responder guinea pigs injected with 4.3 mg of  $H^3$ DNP-poly-L-lysine intraperitoneally. Average and range of values from 4 guinea pigs are plotted.

*Antigenicity of Hapten-Polylysine Conjugates.*—It has been shown that lightly coupled hapten conjugates of poly-L-lysine are antigenic in certain genetically selected guinea pigs (responders) (1-5), whereas lightly coupled benzylpenicilloyl conjugates of poly-L-lysine whose  $\epsilon$ -amino groups have been exhaustively succinylated (4), and lightly coupled benzylpenicilloyl-poly-D-lysine conjugates (5, 6) are non-antigenic even in responder guinea pigs. Since the present study deals with the relationship between antigenicity of various hapten-polylysine conjugates and their abilities to be degraded, we have determined the antigenicities of the hapten-polylysine conjugates used in the present work in responder guinea pigs. Each conjugate was tested in two strain 2 guinea pigs and in two responder random-bred guinea pigs using the immunization and testing methods described previously (2). The fluorescein and  $H^3$ DNP conjugates of PDL and the succinylated  $H^3$ DNP-PLL conjugate

were completely non-antigenic. Two of the 4 animals immunized with 100  $\mu\text{g}$  of succinylated Fluor-PLL in complete Freund's adjuvant gave trace delayed skin reactions and mild anaphylactic symptoms on intravenous injection of the immunizing antigen. None of the 4 gave Arthus skin reactions. Four responders immunized similarly with 100  $\mu\text{g}$  of Fluor-PLL all showed strong Arthus and delayed skin reactions. The low degree of antigenicity of the succinylated Fluor-PLL preparation may have been due to the presence of some conjugate which had not been fully succinylated.

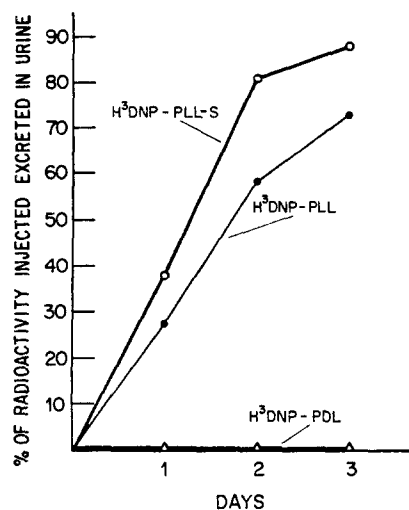


FIG. 4. Cumulative urinary excretion of radioactivity from groups of guinea pigs intraperitoneally injected with 2.0 mg of H<sup>3</sup>DNP-PLL or succinylated H<sup>3</sup>DNP-PLL, or with 1.4 mg of H<sup>3</sup>-DNP-PDL.

#### DISCUSSION

These experiments were designed to determine whether the differences between responder and non-responder guinea pigs could be explained on the basis of their abilities to enzymatically degrade hapten-poly-L-lysine (PLL) conjugates. The *in vivo* experiments show that both responders and non-responders injected with H<sup>3</sup>DNP-PLL excrete in their urines low molecular weight degradation products of the conjugate at equal rates and in equal total quantities. In order to determine whether immunological tissues of responders and non-responders could degrade PLL conjugates to the same extent, aqueous extracts of spleens from both groups were compared with regard to their abilities to degrade the Fluor-PLL conjugate *in vitro*. Paper chromatograms and starch gel electrophoreses of degradation mixtures showed identical fluorescent spots for both groups, indicating the presence of identical



fluorescein-tagged degradation products. These fluorescent spots do not represent hydrolyzed free hapten; *i.e.*, fluorescein thiocarbamic acid. First, several different fluorescent spots were observed by both analytic methods. Secondly, the trace fluorescent impurity in the undegraded Fluor-PDL control, which is probably fluorescein thiocarbamic acid, demonstrated an  $R_f$  different from the  $R_f$  of the 4 fluorescent spots resulting from the degradation of Fluor-PLL (Fig. 1). Furthermore, control experiments rule out the possibility that these spots represent undegraded Fluor-PLL non-specifically bound onto proteins present in spleen extracts. Accordingly, these spots must represent families of fluorescein-poly-L-lysine peptides of different molecular sizes. Direct identification of polypeptide degradation products of poly-L-lysine by ninhydrin staining of the chromatograms could not be carried out because of the intense staining contributed by polypeptide materials in the spleen extracts. Since both responder and non-responder guinea pigs appear to degrade hapten-PLL conjugates into hapten-bearing polypeptides which are indistinguishable by two analytical methods, both groups must possess in their spleens comparable amounts of lysine-specific proteolytic enzymes capable of degrading the poly-lysine carrier.

Although exhaustive succinylation of hapten-PLL conjugates destroys their antigenicity in responder guinea pigs, the succinylated Fluor-PLL and H<sup>3</sup>DNP-PLL conjugates are enzymatically degraded *in vivo* and *in vitro* about as readily as are the unsuccinylated PLL conjugates. No conclusions can be made as to the relative molecular sizes of the degradation products of the succinylated and the unsuccinylated conjugates from the data available at present. In contrast, hapten-poly-D-lysine conjugates which are also non-antigenic in guinea pigs (5, 6) were not degraded *in vitro* or *in vivo*.

It is currently considered that in order to be antigenic, macromolecular substances must possess antigenic determinants, and must be susceptible to enzymatic degradation by immunological tissues. The observation that hapten-poly-D-lysine conjugates, which are not antigenic in guinea pigs, cannot be degraded *in vivo* may be considered to be consistent with the view. However, the present findings that hapten-PLL conjugates, which are non-antigenic in non-responder guinea pigs, and exhaustively succinylated hapten-PLL conjugates, which are non-antigenic even in responder guinea pigs, are both degraded by immunological tissues demonstrates that there is an additional requirement for antigenicity. Since responder and non-responder guinea pigs both degrade hapten-PLL conjugates in an undistinguishable manner, this additional requirement must be for a further metabolic step,<sup>2</sup> which in this

<sup>2</sup> H<sup>3</sup>-DNP conjugates of PLL, succinylated PLL, and PDL all appear in draining lymph node macrophages in comparable amounts following injection into the foot-pads (15). Thus it is unlikely that the difference between responder and non-responder guinea pigs is in their abilities to phagocyte the conjugates.

system is specific for the lysine side chain, and is controlled by a single gene. The nature of this step is unknown. One possibility is that this step may be the coupling of an antigenic fragment to RNA which would require an activating enzyme specific for the lysine side chain.

#### SUMMARY

The enzymatic degradation of fluorescein conjugates of poly-L-lysine, poly-D-lysine, and exhaustively succinylated poly-L-lysine by aqueous extracts of spleens from "responder" (guinea pigs which can develop immune responses to hapten-poly-L-lysine conjugates) and "non-responder" guinea pigs was investigated.

The *in vivo* degradation of H<sup>3</sup>-tagged dinitrophenyl conjugates of these synthetic polyamino acids was also studied by measuring urinary excretion of radioactive low molecular weight degradation products of these conjugates after their intraperitoneal injection.

It was found that both responder and non-responder guinea pigs can degrade succinylated and unsuccinylated poly-L-lysine conjugates into small molecular fragments, but they cannot degrade hapten-poly-D-lysine conjugates.

These studies demonstrate that in addition to the known requirements for antigenicity of macromolecules, *i.e.* the presence of antigenic determinants, and their capacity to be degraded by immunological tissues, the resulting degradation products must undergo certain additional, as yet unidentified, specific metabolic steps in order to induce an immune response.

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