THE EFFECT OF ANTIGENIC STIMULATION ON INCORPORATION OF PHOSPHATE AND METHIONINE INTO PROTEINS OF ISOLATED LYMPH NODE CELLS*

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An important means for analyzing a number of basic immunological problems has been furnished by the discovery that lymphoid cells derived from immunized and sensitized donors will transfer to untreated recipients of the same species the immune and allergic reactivities exhibited by the donor (1, 2). Successful transfer among laboratory rodents has thus far only been accomplished consistently with isolated cells that are intact and presumably viable (3).[1] In regard to serum antibodies, there is also considerable evidence that cell transfer involves the capacity to synthesize antibodies rather than the transfer of antibodies preformed in the cells (4, 5). Accordingly, the effects of antigenic stimulation on the metabolic activities of lymph node cells are of interest in relation to the mechanisms by which these cells participate in immune and hypersensitive reactions.

The present report describes the capacity of isolated lymph node cells to incorporate orthophosphate from the extracellular medium into phosphoproteins. It has been found that the rate of this incorporation is greater in cells taken from lymph nodes of antigenically stimulated guinea pigs than in the corresponding cells of suitable control animals. The rate of incorporation of L-methionine into the same protein fraction is also enhanced in cells isolated from lymph nodes of antigenically stimulated guinea pigs. The time relationship between antigen injection and appearance of these augmented metabolic functions in the isolated cells has been partially characterized.

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[1] Jeter, Tremaine, and Seebohm, working with peritoneal exudate cells, have claimed that cell-free supernates (obtained after sonic disruption of cells and centrifugation at 15,000 *g*) are as effective as intact cells in transferring contact skin sensitivity and tuberculin-sensitivity (26, 27). However, in the case of isolated lymph node cells, about 5×10^8 intact cells are needed, as a rule, for transfer of contact skin sensitivity, but 5×10^9 sonically disrupted cells are ineffectual (24).

Materials and Procedures

The animals used were male guinea pigs of the Hartley strain weighing approximately 350 gm. For each experiment two groups of guinea pigs, with three animals per group, were used. One group was injected with a protein in a water-in-oil emulsion. The second group, hereafter referred to as the control-injected group, received the same quantity of emulsion from which the protein was omitted. Both groups received their respective injections at the same time and their cells were subsequently harvested and compared simultaneously. All injections were made into the footpads in 0.1 ml. volumes. The antigen used was DNP-BγG,[2] a total of 1.7 mg. protein being injected in each animal. In a few experiments, smaller quantities of DNP-BγG were injected, and in a few other experiments DNCB was used in place of DNP-BγG. The emulsion corresponded to the "incomplete" adjuvant of Freund and was made by emulsifying water or an aqueous solution of DNP-BγG with mineral oil (bayol F) and arlacel A (mannide monooleate) in the proportions 5:4:1 by volume, respectively (6). When DNCB was used it was added as a 0.15 M NaCl solution, about 2 per cent in respect to ethanol.

At varying times after footpad injections, an antigen-injected group and its paired control-injected group were sacrificed by an intraperitoneal injection of sodium pentobarbital and exsanguinated. Cells were harvested from popliteal, axillary, and superficial inguinal lymph nodes by procedures which are described elsewhere (7). Cells obtained from the animals of each group were pooled, and on the basis of optical density measurements the cell suspensions from the two groups under comparison were adjusted so as to have the same number and mass of cells per milliliter. All optical density measurements were made with a Beckman spectrophotometer, model DU, using cuvettes with a 1 cm. light path. The validity of this procedure is discussed below. Cell suspensions, in duplicate, were incubated in air at 37°C. with P^{32}-orthophosphate, or with S^{35}-L-methionine, and agitated just often enough to maintain a homogeneous suspension. The detailed composition of each reaction mixture is described in the legends of Tables II and III. Reaction mixtures were inactivated and precipitated by addition of cold TCA to a final concentration of 5 per cent. The precipitates were washed twice with cold 5 per cent TCA, extracted with ethanol-ether 3:1 (*V/V*) at 48°C. for 15 minutes, and treated again with 5 per cent TCA in a boiling water bath for 15 minutes. The precipitates were washed at room temperature once again with 5 per cent TCA, then with ethanol-ether (3:1), and finally with ether. After each extraction the precipitates were collected by centrifugation. The dry precipitate finally obtained corresponds to the "phosphoprotein" fraction as isolated by the procedure of Schneider (8). The dried material was dissolved by heating for 7 minutes in 5.2 M acetic acid in a boiling water bath. One aliquot was dried for the determination of its radioactivity. Other aliquots were analyzed in duplicate for protein by the method of Lowry *et al.* (9). The radioactivity incorporated is expressed as counts per minute per milligram protein of the finally isolated material.

The basic medium used for harvesting and maintaining the cells as well as for the studies on P^{32} incorporation had the following composition: 0.01 M tris, pH 7.4, 0.001 M $MgSO_4$, 0.002 M $CaCl_2$, 0.12 M NaCl, and 0.006 M KCl. When the uptake of S^{35}-L-methionine was studied, the medium used was as above, except that the buffers were 0.005 M tris (pH 7.4), and 0.005 M phosphate (pH 7.4); vitamins and glutamine were added in amounts recommended by Eagle (10).

P^{32}-phosphate was obtained from the Oak Ridge National Laboratory. This material was

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[2] Abbreviations used are as follows: DNCB, 2,4-dinitrochlorobenzene; $DNBSO_3$, 2,4-dinitrobenzenesulfonate, K salt; DNP-BγG, 2,4-dinitrophenyl bovine gamma globulin; BγG, bovine gamma globulin; tris, tris-(hydroxymethyl)amino methane; TCA, trichloroacetic acid.

heated in 1 N HCl in a boiling water bath for 45 minutes to remove acid-insoluble radioisotope (presumably polyphosphates). P^{32} was counted with a thin end-window Geiger tube.

S^{35}-L-methionine was obtained from Abbott Laboratories, Inc., North Chicago. For S^{35} counting, samples were dried on polyethylene planchets (area about 4.9 cm.^{2}) and counted in a flow-gas windowless Geiger counter. Self-absorption corrections were not applied since in each experiment the samples to be compared had essentially the same weight. Sample weights varied in separate experiments from 0.1 to 0.3 mg.

The DNP-BγG used as an antigen was prepared by reacting BγG with $DNBSO_3$, K salt, as previously described (11, 7). Based on optical density measurements in 0.01 N NaOH at 290 mμ and at 360 mμ it was estimated that the conjugated protein had 21 DNP groups per BγG molecule of 160,000 molecular weight (12).

Ascending chromatograms were performed with Whatman 1 paper. Two solvent systems were used: (*a*) butanol, propionic acid, and water in volume ratios of 47:22:32 respectively (13), and (*b*) 95 per cent ethanol, propionic acid, and water in volume ratios of 3:1:1 respectively. After developing for 48 hours, papers were dried, sprayed with Hanes's reagent (14), and irradiated with ultraviolet light (15).

Phosphorus was determined by the method of Fiske and SubbaRow (16).

Eastman Kodak Co., Rochester, New York, furnished DNCB and $DNBSO_3$. The former was recrystallized twice from ether; the latter was recrystallized 4 or 5 times from water as the potassium salt. BγG (fraction II of bovine plasma), crystallized hog pepsin, and crystallized chymotrypsin were products of Armour and Company, Chicago. Crystallized trypsin was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. O-phosphoserine and O-phosphothreonine were purchased from the California Corporation for Biochemical Research.

RESULTS

Quantitation of Cell Suspensions by Optical Density Determination.—The basic procedure employed in this study involved comparisons of suspensions of 2 cell populations (derived from antigen-injected and control-injected animals, respectively) which were initially adjusted so as to have the same optical density. Since antigenic stimulation induces profound histologic changes in regional lymph nodes (17), the validity of this procedure depends upon the demonstration that: (*a*) optical density is a linear function of cell mass, and (*b*) that equal optical densities for the 2 cell populations under comparison correspond to equal protoplasmic masses. That these requirements are met is shown by the data of Figs. 1 and 2.

In Fig. 1, optical density is shown to vary linearly with cell mass up to an optical density of about 0.28. When determinations of packed cell volume were made, by centrifugation at 1300 *g* for 5 minutes in calibrated McNaught centrifuge tubes,[3] the two populations were shown to have indistinguishable optical density/packed cell volume ratios (Fig. 2). In addition, the two cell populations had equal protein concentrations when adjusted to the same optical density.

On the average, 1 ml. of a cell suspension with an optical density (660 mμ)

[3] Calibrated centrifuge tubes of the McNaught type were obtained from the A. S. Aloe Co., St. Louis.

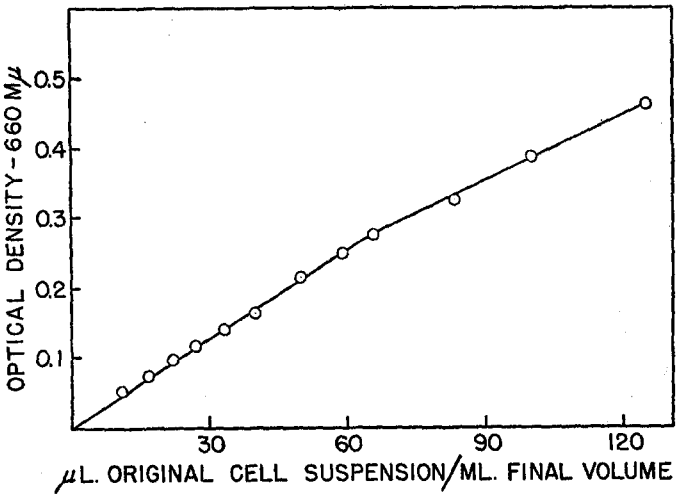

FIG. 1. Linear relationship between cell concentration and optical density. Optical density was measured at 660 mμ with various dilutions of a stock suspension of lymph node cells.

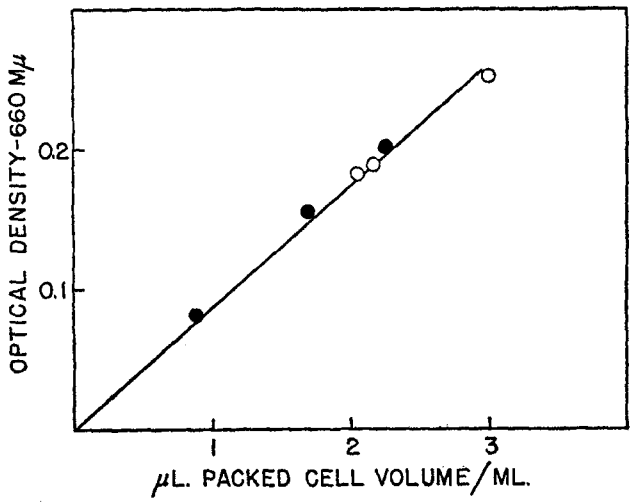

FIG. 2. Relationship between cell volume and optical density. Packed cell volumes, of the order of 100 μl., were determined by centrifugation (see text) of stock cell suspensions, and optical density measurements were made on 30-fold dilutions of the same stock suspensions. Abscissa corresponds to 1/30th of the actual packed cell volumes per ml. of stock suspensions. Filled circles (●) represent cells harvested 4 days after injection of DNP-BγG (1.74 mg.) in "incomplete" adjuvant; open circles (○) represent cells obtained 4 days after injection of "incomplete" adjuvant.

of 0.100 had 2.95×10^6 cells, corresponding to a packed cell volume of 1.15 μl., and to 80 μg. protein (based on a BγG standard), and to 150 μg. dry weight (18).

Characteristics of the Incorporating Activity.—The rate of P^{32} phosphate incorporation into phosphoprotein was constant for at least 3 hours and was proportional to cell concentration over the range 3×10^6 to 3×10^7 cells per ml. P^{32} incorporation was not observed in the presence of excess P^{31} phosphate. The effect of added glucose was small, the rate of P^{32} incorporation being lowered only 30 per cent by omission of this substrate.

When S^{35}-L-methionine was used as a substrate, the rate of S^{35} incorporation into protein was constant for 1 hour, and was linear with respect to cell concentration over the range 1×10^7 to 4×10^7 cells per ml. The omission of glucose reduced the rate of S^{35} incorporation by 60 per cent in the presence of oxygen and by 85 per cent in the absence of oxygen. With glucose present, the rate of incorporation was also reduced by sodium azide (85 per cent inhibition at 10^{-2} M), and by iodoacetate (85 per cent inhibition at 10^{-3} M).

The S^{35} label of S^{35}-L-methionine was incorporated at a rate which was 50 per cent faster than the C^{14} label of C^{14}-methyl-L-methionine. This difference may be due to conversion of some methionine to cysteine, and incorporation of S^{35} cysteine. With C^{14}-methyl-L-methionine the rate of incorporation increased with increase in initial extracellular concentration of L-methionine, reaching a maximum rate of 1 mμmole methionine incorporated per mg. cell protein per hour at an initial extracellular concentration of 2.5×10^{-4} M L-methionine.

P^{32} and S^{35} incorporated at zero time (radioisotope added just before inactivation) were less than 5 per cent of that incorporated after 60 minutes incubation.

Characterization of the Phosphoprotein Product.—Direct assays for tissue phosphoproteins are lacking. The most direct evidence that the TCA-insoluble product studied contains phosphoproteins was derived from (*a*) the effects of proteolytic enzymes, and (*b*) identification of phosphorylated amino acids in acid hydrolysates.

The conversion of P^{32} from a TCA-insoluble to a TCA-soluble form following digestion with proteolytic enzymes is shown in Table I. The results obtained with crystallized pepsin are especially significant since they were obtained at pH 1.5, making it unlikely that the findings were due to other enzymes contaminating the pepsin.

When the TCA-insoluble product was heated for 5 hours in 1 N HCl in a sealed tube at 100°C., about 50 per cent of the acid-soluble P^{32} obtained was chromatographically coincident with authentic O-phosphoserine (Fig. 3 B); with the solvent system used O-phosphoserine and O-phosphothreonine cannot be distinguished. However, with another solvent system (ethanol–proprionic acid–water), these O-phosphorylated amino acids can be distinguished chro-

TABLE I

*Effect of Proteolytic Enzymes on P^{32}-Phosphoproteins**

Fraction	Pepsin		Trypsin + chymotrypsin	
	Experimental	Control	Experimental	Control
	C.P.M.	C.P.M.	C.P.M	C.P.M.
TCA-soluble	1312	70	1498	59
TCA-insoluble	211	1436	244	1740
Per cent soluble	86	5	87	3

* The pepsin test system contained 30 μmoles HCl, 100 μg. crystallized pepsin and P^{32}-phosphoprotein in a total volume of 1.05 ml. The trypsin-chymotrypsin test system contained 100 μg. of each of the crystallized enzymes, 100 μmoles tris (pH 7.4), 5 μmoles $MgCl_2$, and P^{32}-phosphoprotein in a total volume of 1.15 ml. Control vessels did not contain enzyme. All samples were incubated at 37°C. for 1 hour before TCA was added to a final concentration of 5 per cent.

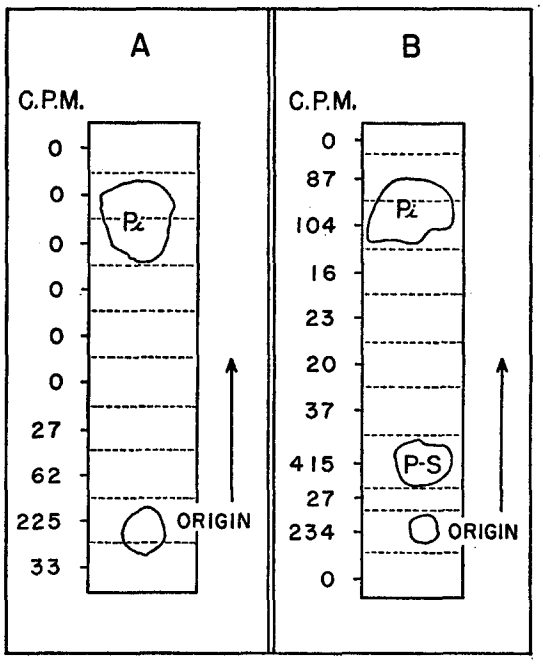

FIG. 3. Paper chromatograms of acid hydrolysates of P^{32}-phosphoproteins isolated from lymph node cells.

A. P^{32}-labelled phosphoprotein fraction was suspended in 1 N HCl and heated at 100°C. for 30 minutes. Inorganic orthophosphate (*Pi*) was added as a carrier to the partial hydrolysate, and an aliquot was applied to paper. The amount applied corresponded to 20 μg. P^{31} phosphate (*Pi*).

B. P^{32}-labelled phosphoprotein fraction was suspended in 1 N HCl in a sealed tube and heated at 100°C. for 5 hours. O-phosphoserine (*P-S*) and inorganic phosphate (*Pi*) were added to the partial hydrolysate and an aliquot was applied to paper. The volume applied contained 20 μg. each of carrier O-phosphoserine and inorganic P^{31}-phosphate.

The solvent used for both chromatograms contained butanol, propionic acid, and water in a volume ratio of 47:22:32, respectively.

matographically on paper. When the hydrolysate was chromatographed with the latter solvent system, acid-soluble P^{32} migrated with the two O-phosphorylated amino acids; in addition there was also one faster moving P^{32} component which probably was a phosphopeptide.

Further justification for designating the P^{32} labelled TCA-insoluble product as "phosphoproteins," rests on the following arguments which exclude other possibilities:

TABLE II

*Effect of Antigen Injection on the Rate of P^{32} Incorporation into Phosphoproteins**

Experiment No.	Time after injection	Guinea pigs injected with		Difference $\frac{A-B}{B} \times 100$
		DNP-BγG§ + adjuvant A	Adjuvant alone B	
	days	*rate*‡	*rate*‡	*per cent*
1	3	1030	1000	+3
2	"	1050	1000	+5
3	"	670	670	0
4	"	680	670	+1
5	"	740	700	+6
6	"	610	650	−6
7	4	1860	1370	+36
8	"	1590	1100	+45
9	"	1280	1010	+27
10	"	590	560	+5
11	"	840	730	+15
12	"	790	660	+20

* Reaction vessels contained 4 to 8 × 10^7 cells, 16.7 μmoles glucose, and approximately 0.5 μcuries P^{32}-phosphate in balanced salt solution with a final volume of 3.0 ml. (see Methods and Procedures). In any given experiment, equivalent quantities of cells and P^{32} were used. Reaction mixtures were incubated at 37°C. for 1 hour before inactivation and preparation for analyses.

‡ C.P.M./mg. protein/hr.

§ Each animal injected with 1.7 mg. protein (1.0 μmole DNP)

(*a*) The method of preparation of the sample excludes at least gross amounts of other phosphorus-containing substances (8). Low molecular weight substances, nucleic acids, and most of the phospholipides are removed by treatment with hot TCA and ethanol-ether, respectively. Phospholipides which contain inositol are not extracted by ethanol-ether, but are removed by treatment with hot TCA (19).

(*b*) The product is not an inorganic polyphosphate since acid hydrolysis (1 N HCl, 100°C.) for 30 minutes does not release P^{32} orthophosphate (Fig. 3 A, *Pi*).

(*c*) Treatment of the TCA-insoluble product with 1 N KOH at 37°C. for 18 hours renders the P^{32} soluble, as is the case with all known phosphoproteins (20). The same result, however, would be expected if the P^{32} were present in the form of RNA. RNA as a major source of the P^{32} would, however, not explain the effect of pepsin at pH 1.5, and the identification of O-phosphoserine and O-phosphothreonine in acid hydrolysates.

TABLE III

*Effect of Antigen Injection on the Rate of S^{35}-Methionine Incorporation into Proteins**

Experiment No.	Time after injection	Guinea pigs injected with		Difference $\frac{A-B}{B} \times 100$
		DNP-BγG§ + adjuvant A	Adjuvant alone B	
	days	*rate‡*	*rate‡*	*per cent*
13	3	1140	1110	+3
14	"	1030	1110	−7
15	"	1020	870	+17
16	"	1030	870	+18
17	"	2720	3370	−19
18	"	2720	2880	−6
19	"	830	970	−14
20	"	970	970	0
21	4	1000	910	+10
22	"	1230	1130	+9
23	"	2840	2480	+15
24	"	4440	3740	+19
25	"	4390	3290	+33
26	"	3010	3010	0

* Reaction vessels contained 4 to 8 × 10^7 cells, 5.5 μmoles glucose, 0.07 μmoles S^{35}-L-methionine (about 90,000 C.P.M.) in a balanced salt solution with a final volume of 1.0 ml. (see Methods and Procedures). In any given experiment, equivalent quantities of cells and S^{35} were added. Reaction mixtures were incubated at 37°C. for 45 to 60 minutes before inactivation and preparation for analyses.

‡ C.P.M./mg. protein/hr.

§ Each animal injected with 1.7 mg. protein (1.0 μmole DNP).

The foregoing evidence indicates then, that the isotopically labelled product finally isolated contains phosphoproteins.

Effects of Antigenic Stimulation on Phosphate Incorporation.—Cells harvested 4 days after the injection of antigen had a distinctly higher rate of P^{32} incorporation into phosphoproteins than the corresponding cells of paired control-injected animals (Table II). One day earlier, however, cells from antigenically stimulated animals and cells from paired controls were indistinguishable in respect to this activity (Table II, 3 days after footpad injections).

P^{32}-incorporating activity was examined under a variety of other conditions and the results are given in Table IV. Six days after footpad injections, P^{32}-incorporating activity was not consistently enhanced in the cells of antigenically stimulated animals. When the amount of DNP-BγG injected was reduced, cells harvested 4 days later from antigen injected and from control-injected animals had essentially the same incorporating activity. When DNCB was injected in an amount sufficient to induce DNP-specific contact skin sensitivity, P^{32}-incorporating activity of cells isolated 4 days later was the same as that of control-injected guinea pigs.

TABLE IV

*Effect of Time and Antigen Variations on P^{32} and S^{35} Incorporating Activities**

Conditions	Differences‡	
	P^{32}-phosphate incorporation	S^{35}-methionine incorporation
	per cent	*per cent*
6 days after injecting 1.7 mg. DNP-BγG (1.0 μmole DNP)	0, +8, +5, +25	−4, +17, +26, +39
4 days after injecting 0.17 mg. DNP-BγG (0.1 μmole DNP)	−4, −9, +6, +12, +22	−6, +2, +4, +11
4 days after injecting 0.1 μmole DNCB	0, 0, +1, +11, −8	−3, −5, −26, +11, +14

* Reaction conditions were the same as for Tables II and III. Each value represents the difference between pooled cells of an antigen-injected group and pooled cells of the paired control-injected group.

‡ $\frac{\text{Rate in cells of antigen-injected animals} - \text{rate in cells from controls}}{\text{Rate in cells from controls}} \times 100$

Rates are C.P.M./mg. protein/hr.

Effects of Antigen Injection on S^{35}-L-Methionine Incorporation.—Data on rates of S^{35}-L-methionine incorporation are summarized in Tables III and IV. In general, S^{35}-incorporating activity was in agreement with P^{32}-incorporating activity as regards differences between cells from antigen-injected guinea pigs and cells from paired controls. The results were, however, somewhat more variable.

Although incorporated P^{32} and S^{35} were found and measured in the same gross fraction, it is obvious that both labels were not necessarily incorporated into the same proteins.

DISCUSSION

The essential finding emerging from the present work is that an increase in the rate of phosphate incorporation into phosphoproteins of lymph node cells occurs at a definite time interval after injection of a sufficient quantity

of antigen. Two features indicate a relationship of this observation to antibody formation: (*a*) the increased rate of incorporation is induced by antigen in a cell population known to have antibody-synthesizing capabilities and (*b*) the enhanced rate becomes manifest 4 days after antigen injection; *i.e.*, at a time when antibodies are ordinarily first detectable. In view of the fact that the molecular transformations involved in antibody synthesis are still matters for speculation, it is not possible to consider in any concrete sense relationships between phosphoprotein synthesis and antibody formation. However, a number of possibilities merit discussion.

Since the injection of antigen produces an increase in cell mass of regional lymph nodes, the question may be raised that increased phosphoprotein synthesis is simply a general property of rapidly proliferating cells, regardless of whether or not antibody is being formed. This possibility does not seem to offer a likely explanation for the present results for two reasons:—(*a*) the increase in rate of P^{32} incorporation occurred on the 4th day after antigen injection, whereas lymph node mass increases nearly linearly beginning at the time of antigen injection (21, 22); (*b*) control-injected animals, which did not receive antigen, had, during the time intervals examined, an increase in their regional lymph node mass which appeared to be the same as that of the antigen injected guinea pigs.

If the increased phosphoprotein synthesis in antigenically stimulated lymph node cells is directly related to antibody formation, the simplest explanation would be that antibody itself is a phosphoprotein. This possibility is unlikely because it was found that a specific immune precipitate containing 43 mg. rabbit antibody released no inorganic phosphate after incubation in 1 N KOH for 18 hours at 37°C. Under these conditions, phosphoproteins release esterified phosphate as inorganic orthophosphate (20). From the sensitivity of the method used for phosphorus analysis, there is, therefore, less than one esterified phosphate group per 4 molecules of rabbit serum antibody (molecular weight 160,000). It should be noted, moreover, that esterified phosphate is absent in human and horse gamma globulins (23) and in bovine gamma globulin (24). It is improbable, therefore, that guinea pig serum antibodies contain phosphate groups.

Antigenically induced phosphoprotein synthesis and antibody formation could be directly related to each other in that a precursor of serum antibodies might exist within antibody-synthesizing cells as a phosphoprotein. This possibility is of interest in relation to so-called "cell-fixed" antibodies which have long been believed to mediate delayed type allergic reactions. One might speculate, for example, that "cell-fixed" antibodies are phosphoproteins in which phosphate, through diester linkages, serves to bind antibodies, with full serologic specificity, to insoluble cell structures, and that hydrolysis of the phosphate bonds yields free antibody. Significant experimental support for this speculation does not exist. There are, however, two observations

which are consistent with this concept: (*a*) following sonic disruption of isolated lymph node cells, approximately 95 per cent of the phosphoprotein is sedimentable by centrifugation at 100,000 *g* for 1 hour; (*b*) antibodies contain an extraordinary abundance of serine and threonine (25). The hydroxyl groups of these amino acids are known to function as sites for phosphate esters in phosphoproteins (20). Not consistent with this speculation is the fact that increased P^{32} incorporation was not observed when DNCB was injected in an amount sufficient to induce DNP-specific delayed contact skin sensitivity (0.1 μmole). It should be noted, however, that when DNP-BγG was injected in an amount containing an equivalent quantity of dinitrobenzene (0.1 μmole) the increase in rate of P^{32} incorporation was also not observed (Table IV). Possibly, isolation of lympl node cells at different times after these injections would have revealed increased P^{32} incorporation. It may be noted, moreover, that DNCB is a protein reagent with considerable intrinisic cytotoxic activity. Even at low concentrations, DNCB profoundly inhibits many metabolic activities (18), including P^{32} incorporation (24). This effect of DNCB may interfere with the recognition of metabolic changes which occur when this substance induces hypersensitivity.

Whether or not intracellular antibody exists at some point in time as a phosphoprotein, the speculation that antibodies may be bound through conventional bonds to cellular structures suggests approaches for future investigation of "cell-fixed" antibodies at the molecular level.[4]

SUMMARY

Isolated lymph node cells incorporate inorganic orthophosphate into a protein fraction. The phosphorylated product is a phosphoprotein. The rate of phosphate incorporation into phosphoprotein was determined in cells isolated from regional lymph nodes at varying times after antigen injection. The rate was unaltered on the 3rd day, but was enhanced on the 4th day after injection. Parallel results were obtained with L-methionine incorporation into the same gross protein fraction. Possible relationships between antibody formation and the observed enhancement in phosphate incorporation into phosphoprotein are discussed.

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