Effect of Antibody–Complement on Multiple *vs.* Single Compartment Liposomes. Application of a Fluorometric Assay for Following Changes in Liposomal Permeability†

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ABSTRACT: Multiple compartment (MC) and single compartment (SC) liposomes were prepared from a mixture of sphingomyelin, cholesterol, dicetyl phosphate, and dinitrophenylaminocaproylphosphatidylethanolamine (as antigen). An immunodiffusion procedure is described that supplements more conventional methods for identifying SC liposomes. As shown previously by a spectrophotometric assay, MC liposomes release 60–70% of their trapped glucose when incubated with both anti-dinitrophenyl antibodies and guinea pig serum (as complement source). Under comparable conditions, however, glucose release from SC liposomes was not obtained, and the present investigation describes the limitations of the spectrophotometric assay that are responsible. A more sensitive fluorometric assay for detecting changes in liposomal permeability was therefore developed which uses a fluorogenic substrate, umbelliferone phosphate, as trapped marker. With this assay, it has been demonstrated that antibody–complement produce essentially 100% marker release from SC liposomes. These results support the earlier hypothesis that incomplete marker loss is probably due to an inability of cytolytically active complement components to reach the innermost lipid bilayers. Thus, SC liposomes may be more suitable than MC liposomes for investigating the mechanism by which the complement system causes membrane damage.

Sheep erythrocytes have long been the favored target cell for studying immune cytolysis by antibody–complement because the terminal event can be easily followed by the appearance of hemoglobin in the medium. Previous investigations (reviewed in Kinsky, 1972) have shown that antibody–complement will also promote release of trapped glucose marker from liposomal model membranes, sensitized with an appropriate amphipathic antigen, in a manner that closely parallels erythrocyte hemolysis (see *e.g.*, Haxby *et al.*, 1969). For this reason, liposomes have been employed to investigate certain aspects of complement action with particular emphasis on the question of whether immune damage occurs by an enzymatic mechanism involving covalent bond cleavage in membrane lipids.

However, as discussed in detail elsewhere (Inoue and Kinsky, 1970; Kinsky, 1972), hemoglobin and glucose release are not strictly comparable phenomena because of certain morphological differences between erythrocytes and liposomes. Erythrocytes may be regarded as a single aqueous compartment, bounded by the cell membrane, which contains a high concentration of a large charged macromolecule (*i.e.*, hemoglobin). Thus, it is now generally accepted that immune lysis of these cells proceeds by a colloid-osmotic mechanism due to the formation of membrane lesions which are initially too small to permit the escape of hemoglobin but of sufficient size to allow the exchange of cations. Such lesions are presumably also responsible for the loss of glucose from liposomes but, in this regard, the following must be emphasized: the model membranes that we have examined so far are multiple compartment structures in which alternating arrays of lipid bilayers separate aqueous regions that contain only the trapped low molecular weight marker. In the presence of excess antibody and complement, the amount of glucose released from these MC liposomes[1] rarely exceeds 70% of the quantity trapped; this was tentatively attributed to a lack of immune damage to the innermost lipid bilayers (*cf.* Kinsky, 1972; Uemura and Kinsky, 1972) because it contrasts markedly with the response of erythrocytes which can be lysed completely.

To test this hypothesis, we examined the immunological sensitivity of single compartment liposomes and found, to our surprise, essentially no release of trapped glucose utilizing the spectrophotometric assay employed successfully in previous studies with MC liposomes. The latter is based on the increase in absorbancy at 340 nm occurring when any released marker is rapidly oxidized with the concomitant production of TPNH in the presence of hexokinase, glucose-6-phosphate dehydrogenase, and the requisite cofactors. Subsequent experiments (described below) revealed that this spectrophotometric procedure possessed several serious disadvantages when applied to SC liposomes prepared from MC liposomes by prolonged sonication. In this connection, it is also significant that Knudson *et al.* (1971) found that this treatment of liposomes rendered them insensitive to antibody–complement as indicated by a spectrophotometric assay whose basis was the release of trapped galactose.[2]

It seemed to us particularly important to determine whether SC liposomes are indeed resistant to immune damage because numerous laboratories have exploited these model membranes to examine the orientation, packing, mobility, etc., of lipids in bilayers by physical techniques such as transition temperature analysis and nuclear magnetic and electron spin resonance spectroscopy. This has immediate relevance to the problem of

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[1] Multiple compartment and single compartment liposomes are designated MC and SC, respectively. Abbreviations used are: Dnp-Cap-PE, 2,4-dinitrophenylaminocaproylphosphatidylethanolamine (see structure in Six *et al.*, 1973); UmP, umbelliferone phosphate.

[2] After submission of this manuscript, Humphries and McConnell (1974) reported failure to obtain release of a spin-label marker from sonicated liposomes in the presence of antibody and complement; the spin-label assay is apparently more sensitive than either of the spectrophotometric procedures.

immune lysis because our previous liposomal investigations suggest that an enzymatic mechanism is probably not involved in the final step (Inoue and Kinsky, 1970; Kinsky *et al.*, 1971); instead, it has been proposed that activation of the terminal complement components results in the exposure (or liberation) of hydrophobic regions in these proteins which then disrupt the noncovalent bonds that are responsible for maintaining lipids in stable bilayer configuration. These considerations prompted the present efforts to develop an extremely sensitive assay that would require small quantities of SC liposomes to detect any changes in their permeability characteristics. Attainment of this objective was achieved by trapping a fluorogenic substrate (umbelliferone phosphate) into the aqueous liposomal compartment; its release was followed after conversion by alkaline phosphatase into an intense fluorophor (umbelliferone). With this assay, we have been able to show that antibody–complement produces essentially complete release of the marker from SC liposomes.

Experimental Section

Immunologic Reagents. The rabbit IgG anti-dinitrophenyl antibodies were the same high affinity preparations (K_0 for ϵ-Dnp-lysine of 10^8 l. mol^{-1}) used in earlier investigations (Uemura and Kinsky, 1972; Six *et al.*, 1973). The complement source was guinea pig serum that had been extensively dialyzed against a cold buffer solution (containing 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$) to reduce endogenous glucose levels; it should be noted that this solution (hereafter referred to as complement buffer) replaces the Veronal-buffered saline which had been previously employed for this purpose (Kinsky *et al.*, 1969).

Lipids. Beef brain sphingomyelin, cholesterol, and dicetyl phosphate were obtained from the companies listed in Kinsky *et al.* (1969); dinitrophenylaminocaproylphosphatidylethanolamine was synthesized as described in Six *et al.* (1973).[1] The mixture used for generation of liposomes contained these compounds in molar ratios of 2 (sphingomyelin):1.5 (cholesterol): 0.2 (dicetyl phosphate):0.1 (Dnp-Cap-PE); either chloroform or chloroform–methanol (1:1) was the solvent.

Preparation of MC Liposomes. Complete details may be found in Kinsky *et al.* (1969); the following is a brief summary of the method. An aliquot of the lipid mixture, containing 20 μmol of sphingomyelin, was added to a small conical flask and the solvent removed by rotary evaporation. The thoroughly dried lipid film, coating the flask walls, was then dispersed in 1 ml of the appropriate marker solution (see below) to yield a 20 mM phospholipid suspension (note that in earlier investigations, the phospholipid concentration was 10 mM). A portion of these MC liposomes was removed for subsequent conversion into SC liposomes; the remainder of the preparation (usually 0:2 ml) was dialyzed for 90 min against two changes of 125 ml of an isotonic salt solution (75 mM KCl–75 mM NaCl) to remove most of the untrapped marker. Under these conditions, MC liposomes are obtained which have been previously designated as actively sensitized with 5% antigen (Uemura and Kinsky, 1972; Six *et al.*, 1973), *i.e.*, 50 nmol of Dnp-Cap-PE added per μmol of liposomal sphingomyelin.

The marker solutions were either 300 mM glucose, 100 mM umbelliferone phosphate (disodium salt purchased from Isolab, Inc., Akron, Ohio), or mixtures of these compounds that had the same tonicity (*e.g.*, 150 mM glucose–50 mM UmP).[1] Boiled water was used to prepare solutions containing UmP because we were able to confirm the observation of Pitts and Askari (1971) that this precaution effectively reduces spontaneous hydrolysis.

Preparation of SC Liposomes. All steps (adapted from Huang, 1969) were performed at 2°. Undialyzed MC liposomes (approximately 0.7 ml) were sonicated for 2 hr under a nitrogen atmosphere with the microprobe of either a Biosonik II (operating at a power setting of 20) or a Branson Sonifier Model LS-75 (operating at a scale setting of 1 with 1 amp output). The suspension was then centrifuged at 18000*g* for 15 min and the supernatant solution transferred to a Sepharose 4-B column (0.9 × 59 cm) that had been previously coated with phospholipid (Kataoka *et al.*, 1973) and extensively washed with 150 mM NaCl–20 mM Tris (pH 8). The same buffer (hereafter referred to as Tris-saline) was employed for elution and fractions were monitored at a wavelength (350 nm) that would detect light scattering by the liposomes as well as absorbance by the Dnp chromophore and UmP (if present). A representative elution profile is depicted in Figure 1; evidence that peak II contains SC liposomes is presented in the Results section.

Trapped UmP Content of Liposomes. By definition, the amount of any marker trapped in a liposome preparation is the difference between the total and untrapped levels. In the case of UmP, these parameters were determined after enzymatic hydrolysis of this compound into phosphate and the fluorophor, umbelliferone. For this purpose, we employed *Escherichia coli* alkaline phosphatase (Type III enzyme purchased from Sigma Chemical Co., St. Louis, Mo.; approximately 25 U/mg protein, 12 mg of protein/ml). Stock solutions of the enzyme were prepared by extensive dialysis against cold Tris-saline to remove ammonium sulfate and subsequent adjustment of the protein concentration to 5 mg/ml.

Fluorescent measurements were made with a Farrand Ratio Fluorometer (Farrand Optical Co., New York, N. Y.) using a primary filter of Corning glass 4600 and 5840 and a secondary filter of Corning glass 3387 and 5030. The slit was maintained at a setting of 2 and the sensitivity range was varied as required between settings of 0.3–100. The instrument was calibrated with umbelliferone (Aldrich Chemical Co., Milwaukee, Wis.); standards were prepared by tenfold serial dilutions with Tris-saline of a stock ethanolic solution (10 mM umbelliferone). Under these conditions, a linear response was obtained over the desired concentration range between 10^{-9} and 10^{-5} M.

To determine total UmP (basic procedure), aliquots of the liposomes were dispensed into matched fluorometer tubes (75 × 10 mm). Liposomal structure was then destroyed by the addition of 500 μl of chloroform and the tube contents were taken to dryness under a stream of nitrogen. The residue was resuspended in 996 μl of Tris-saline and the fluorescence measured against the appropriate standards before, and approximately 10 min after, the addition of 4 μl of the dialyzed alkaline phosphatase solution (20 μg of enzyme). The difference between the final and initial values corresponded to the total UmP in the sample taken for assay. The latter was contingent on the initial concentration of UmP in the marker solution used to generate the liposomes. Thus, when the liposomes were prepared with 100 mM UmP, we usually assayed 0.4–2.0 μl of MC liposomes or 4–20 μl of SC liposomes; proportionally larger aliquots were employed when the liposomes were formed in the presence of lower UmP concentrations.

To determine untrapped UmP, the preceding steps were repeated under conditions in which the model membranes remained intact, *i.e.*, the liposomal samples were directly diluted with sufficient Tris-saline to give a volume of 996 μl. Again, readings were taken before and after addition of the enzyme, and the difference in these values was calculated as a measure of marker not included in the aqueous compartments of the liposomes.

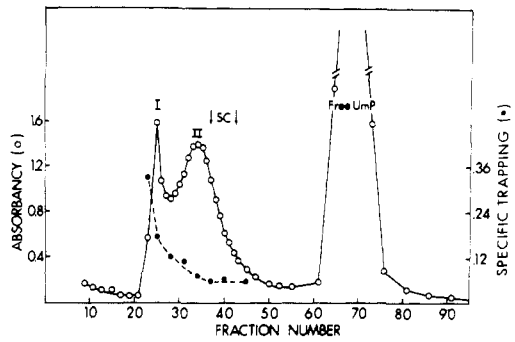

FIGURE 1: Resolution of SC liposomes by Sepharose chromatography. MC liposomes were prepared in a swelling solution that contained 100 mM UmP, and sonicated and centrifuged prior to column application, as described in the Experimental Section. Light scatter or absorbancy was measured at 350 nm to localize fractions (*ca.* 700 μl per tube) that contained liposomes or free UmP (O). Liposomal fractions were also assayed for phospholipid and trapped UmP content (determined by the basic procedure) to calculate specific trapping, *i.e.*, moles of marker trapped per mole of liposomal phospholipid (●). Fractions 37–42 (between arrows) were subsequently pooled as source of SC liposomes.

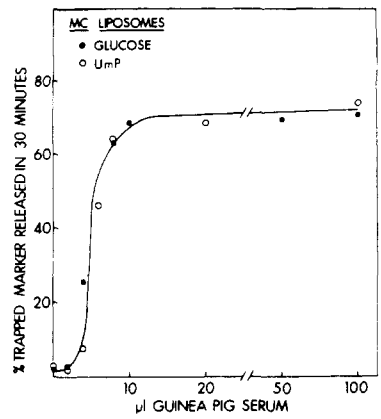

FIGURE 2: Parallel release of glucose and UmP from MC liposomes. MC liposomes were prepared in a swelling solution that contained 285 mM glucose and 5 mM UmP. Cuvets initially contained 500 μl of complete (or incomplete) glucose reagent, the quantities of guinea pig serum indicated in the figure, 6 μl (36 μg) of antibody, and sufficient complement buffer to give a volume of 997 μl; the reaction was started by the addition of 3 μl of liposomes. The steps involved in calculating the per cent trapped glucose released are discussed in connection with the experiment described in Figure 4. To determine the amount of UmP released after 30 min, 100-μl aliquots from each of the incomplete cuvets were added to fluorometer tubes containing 896 μl of Tris-saline and 4 μl (20 μg) of alkaline phosphatase. Following further incubation for 10 min, fluorescence was measured against either a 10^{-6} or 10^{-7} M umbelliferone standard and corrected for the reading produced by the appropriate dilution of guinea pig serum in Tris-saline.

In regard to the above basic procedure, several points merit further comment. (1) Although the tubes were incubated for 10 min after addition of alkaline phosphatase, this time interval was not essential and only included to permit multiple analyses; complete hydrolysis of UmP (initial concentrations: 10^{-9}–10^{-5} M) occurred in less than 1 min at room temperature. (2) The untrapped UmP content of MC and SC liposome preparations (immediately after dialysis and Sepharose chromatography, respectively) is generally less than 5% of the total UmP content. Nevertheless, we routinely assay for this parameter to ensure that untrapped marker was effectively removed. Moreover, the untrapped UmP levels increase slightly over a period of hours (MC liposomes) or days (SC liposomes) so that periodic measurement is considered advisable. Liposome preparations, which contain more than 15% of their total UmP as untrapped marker, are discarded. (3) In both the total and untrapped UmP determinations, initial readings (*i.e.*, before alkaline phosphatase) were taken to correct for such variables as light scatter by the liposomes. Usually, these values were considered insignificant because they amounted to approximately 1% of the fluorescence obtained after enzyme addition. However, preliminary experiments, which led to the eventual development of a fluorometric assay for following immune damage of liposomes (see below), revealed several complications introduced by the complement source. First, at high concentrations, guinea pig serum *per se* gave appreciable readings that could not be ignored; second, the serum partially quenched the fluorescence of umbelliferone; third, stable initial values were unobtainable due to serum alkaline phosphatase that could not be completely inhibited without destroying complement activity.

Accordingly, the basic procedure employed to measure trapped UmP was amended in the following manner. For determination of total UmP, the dried liposomal residue was dispersed in a volume of guinea pig serum equivalent to that present in the immune release assay (up to 125 μl depending on the particular experimental requirements) plus 4 μl of dialyzed alkaline phosphatase and sufficient Tris-saline to yield 1 ml. For measurement of untrapped UmP, the liposomes were mixed with the same quantities of serum, enzyme, and the requisite volume of buffer to give 1 ml. Fluorescence was read after 10 min at room temperature and corrected for the value produced by the appropriate dilution of guinea pig serum in Tris-saline. It was not necessary to add guinea pig serum to the umbelliferone standards because, as in previous investigations, the effect of antibody-complement on liposomal permeability is expressed as the "per cent of trapped marker released." This modified method for determining trapped UmP was validated by experiments showing a linear relationship between the amount of liposomes assayed and total, or untrapped, marker.

Fluorometric Assay for Immune Release of Trapped UmP from SC Liposomes. Fluorometer tubes initially contained the following components: Tris-saline supplemented with 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$, *i.e.*, complement buffer at pH 8 (sufficient to give a volume of 980 μl before the addition of liposomes), the desired amount of antibody (in a volume not exceeding 10 μl), the desired volume of guinea pig serum (not exceeding 125 μl), and 4 μl of dialyzed alkaline phosphatase. Zero time fluorescence was determined and the reaction started by the addition of 20 μl of SC liposomes (pooled fractions from peak II shown in Figure 1); after 30 min at room temperature, the tubes were again read. Under these conditions, the increase in fluorescence was a measure of UmP released from the liposomes plus untrapped marker originally present in the model membrane preparation. Correction for the latter parameter (determined as described above by the modified procedure) permitted calculation of the per cent of trapped UmP released by the immunologic reagents.

Trapped Glucose Content of Liposomes. The spectrophotometric methods used in this laboratory to measure total, untrapped, and (by difference) trapped glucose in liposomes are the subject of a recent review (Kinsky, 1975). Previous experiments that validate these procedures are described in Kinsky *et al.* (1969) (see Kinsky *et al.*, 1970, for correction of a typographical error). At concentrations encountered in this investigation, neither UmP nor umbelliferone interfered with the enzymatic assay which employed the following solutions (identical with those used in all of our studies). The complete glucose reagent contained 100 mM Tris (pH 7.5), 1 mM TPN, 2 mM ATP, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 64 mM NaCl, and ap-

proximately 80 μg of dialyzed hexokinase and 40 μg of dialyzed glucose-6-phosphate dehydrogenase per milliliter (see Kinsky *et al.*, 1969, for sources of enzymes and coenzymes); the incomplete reagent had a similar composition except that TPN was omitted.

Spectrophotometric Assay for Immune Release of Trapped Glucose from MC Liposomes. Kinsky (1975) should also be consulted for details of this procedure; its specific application to liposomes sensitized with dinitrophenylated phosphatidylethanolamine derivatives is illustrated in Uemura and Kinsky (1972) and Six *et al.* (1973). The only modification introduced in the present experiments is the substitution of the complement buffer for Veronal-buffered saline (see "Immunologic Reagents"). Extension of this procedure to permit measurement of the parallel release of glucose and UmP from MC liposomes is described in the legend to Figure 2. Unpublished experiments have shown that, in the presence of excess antibody and complement, the per cent of trapped glucose released from MC liposomes is independent of the quantity of liposomes taken for assay. It is, of course, essential that neither the capacity of the complete glucose reagent nor the capabilities of the spectrophotometer be exceeded. The volume of reagent added to the cuvets contains sufficient TPN to oxidize 500 nmol of glucose which should produce a 340-nm absorbancy increase of 3.125 under our conditions. We employ a Gilford Model 240 spectrophotometer with linear absorbance output to 3.000 although, in practice, the experiments are designed so that the final absorbancy is not greater than 2.8. Further discussion is deferred to the Results section because, as noted in the introduction, it was the limitations of this method when applied to SC liposomes that largely prompted the current investigation.

Miscellaneous. Phospholipid concentration was determined by minor modification of the procedure used by Gerlach and Deuticke (1963) for total phosphate analysis. Immunodiffusion experiments were performed as described in the legend to Figure 3.

Results

Localization of UmP in MC Liposomes. The experiment described in Table I was designed to determine whether UmP could be employed as a trapped marker for following changes in liposomal permeability. In this experiment, MC sphingomyelin–cholesterol–dicetyl phosphate liposomes were formed by swelling the dried lipid films in solutions of constant tonicity but varying concentrations of glucose and/or UmP. The actual amounts of each marker present in the dialyzed liposomes were subsequently normalized to correct for the initial concentration differences in the swelling solutions (see table legend for representative calculation). The values in the last two columns clearly indicate that glucose and UmP are trapped in parallel; analogous data (not shown) have been obtained with MC lecithin–cholesterol–dicetyl phosphate liposomes. These results therefore demonstrate that UmP, like glucose, is localized in the aqueous liposomal compartments.

This conclusion is further supported by the fact that both glucose and UmP are released in parallel from MC liposomes that have undergone immune damage in the presence of varying amounts of guinea pig serum (Figure 2); similar results (not shown) were obtained when antibody concentration was varied. These observations are also particularly important because they indicate that UmP is lost from the model membranes *via* the same "route" responsible for glucose release and thus justify use of UmP as a marker for detection of liposomal permeability alterations induced by antibody–complement.

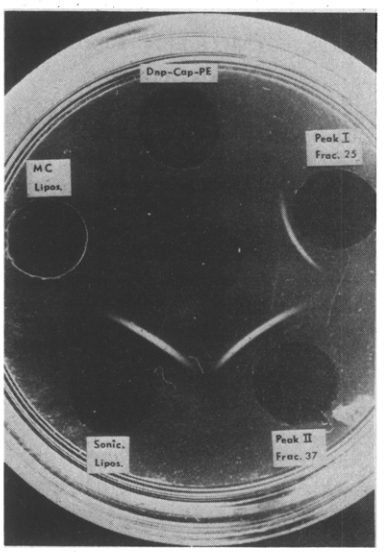

FIGURE 3: Immunodiffusion analysis of liposome preparations. Petri plate (35 mm diameter) prepared with 2 ml of 0.8% agarose in 150 mM NaCl–20 mM Tris (pH 8); center-to-center distance between wells was 10 mm. The central well contained 30 μl (7.5 μg) of antibody. Peripheral wells (see figure) contained 1.5 nmol of Dnp-Cap-PE, or approximately 24 nmol of liposomal phospholipid, in 30 μl of Tris-saline. The liposomes were prepared by swelling in 300 mM glucose, the sonicated liposomes were centrifuged prior to addition, and fraction (peak) numbers correspond to those indicated in Figure 1. The photograph was taken after 20-hr incubation in a humidified chamber at room temperature; the light ring around the MC liposome well is due to light scatter. See text for further discussion.

TABLE I: Parallel Trapping of Glucose and UmP in MC Liposomes.[a]

Composition of Swelling Solution (mM)		nmoles of Marker Trapped (per 5 μl of Liposomes)		nmoles of Marker Trapped (Normalized Values)	
Glucose	UmP	Glucose	UmP	Glucose	UmP
300	0	317.8		318	
225	25	216.4	23.5	288	282
150	50	145.1	42.5	290	255
75	75	53.3	53.9	213	216
0	100		93.5		280

[a] MC liposomes were prepared in swelling solutions that contained glucose and/or UmP at the concentrations specified in the table. After dialysis, the liposomes were assayed for trapped marker content (UmP was determined by the basic procedure). These values were normalized to what the trapping would be if the concentrations of both markers in the swelling solutions had been 300 mM. For example, in the case of liposomes generated in 150 mM glucose–50 mM UmP, the amount of glucose trapped was multiplied by 2 and the amount of UmP trapped was multiplied by 6. See text for further discussion.

Resolution of SC Liposomes by Sepharose Chromatography. In the original description of SC liposome preparation, Huang (1969) generated these model membranes solely from egg lecithin and observed an elution pattern identical with

TABLE II: Comparison of MC and SC Liposomes.[a]

Parameter	MC Liposomes	SC Liposomes	
	Glucose marker	Glucose marker	UmP marker
nmoles trapped/ μl of liposome	92.1	0.141	0.049
nmoles PL/μl of liposome	21.4	0.555	0.67
Specific trapping	4.3	0.25	0.073
Contained volume	14.3	0.83	0.73

[a] Liposomes were prepared in swelling solutions that contained either 300 mM glucose or 100 mM UmP. In the case of SC liposomes with UmP as marker, liposomal phospholipid (PL) was determined as the difference between total phosphate and the amount of trapped UmP (measured by the basic procedure).

those we have now obtained with sphingomyelin–cholesterol–dicetyl phosphate liposomes (Figure 1). This pattern was the same regardless of whether glucose or UmP was present as marker. In view of the preceding experiments demonstrating that both markers are trapped in the aqueous regions of liposomes, we assayed column fractions for phospholipid and UmP (or glucose) content to calculate specific trapping, *i.e.*, moles of marker trapped per mole of phospholipid. Figure 1 shows that specific trapping decreases throughout peak I to a constant value on the backside of peak II. Because constant specific trapping also indicates constant volume (see below), fractions between the arrows in Figure 1 were pooled and used as the source of SC liposomes in all subsequent experiments. Huang characterized the vesicles in peak II (after rechromatography on Sepharose 4-B) as a homogeneous population of SC liposomes on the basis of their physical properties and morphological appearance. Electron microscopic examination of the pooled fractions (after negative staining with sodium phosphotungstate) yielded pictures (not presented) that were indistinguishable from that published by Huang.

Immunodiffusion Analysis of Liposome Preparations. The above findings, and the fact that immunodiffusion has been extensively employed to characterize lipid antigens (see, *e.g.*, Siddiqui and Hakomori, 1971), led to the development of a procedure for identifying column fractions containing SC liposomes; this proved to be a useful adjunct to the other methods (*i.e.*, specific trapping determination, electron microscopy). Figure 3 shows that MC liposomes are unable to diffuse into an 0.8% agarose gel, as evidenced by the absence of a precipitin line, in contrast to the behavior of either a peak I fraction (no. 25 in Figure 1) or a peak II fraction (no. 37 in Figure 1). It should be emphasized that the liposomes in fraction 25 did not migrate as far from the well as those in fraction 37 because this demonstrates an inverse correlation between diffusion distance and specific trapping (note that Figure 1 shows that fraction 25 contains three times more marker than fraction 37). Before resolution by Sepharose chromatography, sonicated liposomes only yielded a precipitin line that corresponded to the one produced by the SC liposomes in fraction 37; the inability of a sufficient quantity of antibody to cross this boundary probably accounts for the failure to detect in the sonicated preparation a line corresponding to fraction 25. In addition, it should be noted that neither MC, sonicated, nor SC liposome preparations yielded precipitin lines when the model membranes were formed without antigen (not shown). Furthermore, as illustrated in Figure 3, we were unable to detect a precipitin line with free antigen, *i.e.*, Dnp-Cap-PE not incorporated into liposomes. Although this could be due to the fact that Dnp-Cap-PE is monovalent, it seems equally likely that the compound exists predominately in the form of micelles that are too large to enter the agarose gel under the present experimental conditions. The latter alternative would be consistent with previous studies on the ability of various dinitrophenylated phosphatidylethanolamine derivatives to either actively or passively sensitize liposomes to immune damage (Uemura and Kinsky, 1972), their effectiveness as immunogens in eliciting an antibody response (Uemura *et al.*, 1974), as well as their precipitability from aqueous suspension by anti-dinitrophenyl antibodies (unpublished observations).

Size of SC Liposomes. As implied above, the more facile diffusion of SC liposomes into, and within, agarose reflects a correspondingly smaller volume than MC liposomes (or the multicompartment liposomes in peak I that have not been reduced to the ultimate size by sonication). The relative dimensions of the aqueous compartments in these liposome preparations can be readily computed from their specific trapping and the initial concentration of marker in the swelling solution. Table II shows that SC liposomes have an average contained volume of 0.78 μl/μmol of phospholipid which is approximately 20 times smaller than that possessed by MC liposomes (note that this difference may be even greater because we have often prepared MC sphingomyelin–cholesterol–dicetyl phosphate liposomes whose specific trapping approaches 6).

In this regard, it should also be noted that our data confirm and extend the observations of Johnson (1973) on SC liposomes generated from egg lecithin and varying amounts of phosphatidic acid and cholesterol. Using mixtures of radioactive glucose, sucrose, KCl, and NaCl, she demonstrated that the cation:sugar volume ratio was 1 provided that the mole per cent of charged amphiphile (*i.e.*, the amount of phosphatidic acid relative to lecithin) was less than 33%; we employed 10 mol % dicetyl phosphate instead of phosphatidic acid and determined that the contained volume in the SC liposomes was essentially the same for glucose and UmP (Table II). Even more important in the context of the present investigation (see Discussion), Johnson demonstrated that incorporation of cholesterol and phosphatidic acid significantly increased the contained volume of SC liposomes from 0.26 μl/μmol of phospholipid (in the case of pure lecithin liposomes) to 0.82 μl/μmol of phospholipid (in the case of liposomes prepared with lecithin, cholesterol, and phosphatidic acid in molar ratios of 1:1:0.2, respectively); the latter is in excellent agreement with the value of 0.78 μl/μmol of phospholipid for liposomes of roughly comparable composition. Kornberg and McConnell (1971) have calculated that spherical vesicles with dimensions indicated by Huang (1969) (*i.e.*, outer diameter of 250 Å with bilayer thickness of 50 Å) should have a contained volume of 0.25 μl/μmol of phospholipid as has been found experimentally (*cf.* discussion in Johnson, 1973). Accordingly, our SC liposomes must be larger and probably have an external diameter closer to 360 Å as determined by Johnson for the lecithin–cholesterol–phosphatidic acid liposomes.

Limitations of the Spectrophotometric Assay. Figure 4 illustrates the basis of the assay employed in all of our previous studies on MC liposomes. Cuvets are set up that initially contain buffer, guinea pig serum, antibody, and either complete (curves 1 and 3) or incomplete (curves 2 and 4) glucose reagents (see figure legend for exact amounts). When the com-

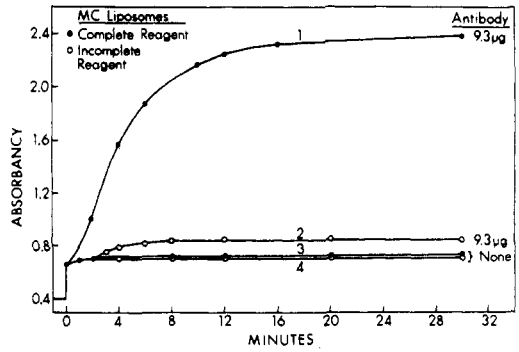

FIGURE 4: Application of the spectrophotometric assay to MC liposomes. Cuvets initially contained 500 μl of complete (●) or incomplete (○) glucose reagent, 125 μl of guinea pig serum, the amounts of antibody indicated in the figure, and sufficient complement buffer to give a volume of 996 μl. Absorbancy was recorded and the reaction started at zero time with 4 μl of MC liposomes (prepared in a swelling solution of 300 mM glucose). Subsequent measurements were made with a recording spectrophotometer and experimental points derived after correction for dilution and any slight variation in cuvet blanks. See text for further discussion.

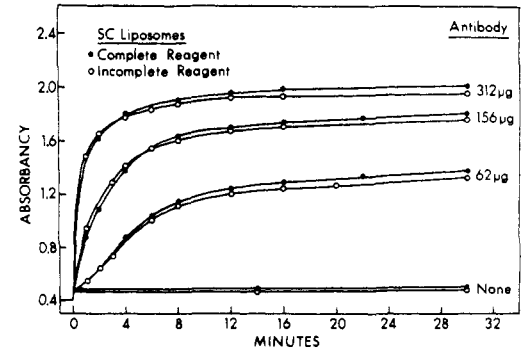

FIGURE 5: Application of the spectrophotometric assay to SC liposomes. Procedure identical with that described in the legend to Figure 4 except for the following: the initial volume (before liposome addition) was 800 μl and the reaction was started with 200 μl of SC liposomes (prepared in a swelling solution of 300 mM glucose).

plement source has been well dialyzed, the absorbancy is the same for complete and incomplete cuvets indicating removal of all endogenous glucose. The reaction is started by the addition of liposomes which results in an immediate absorbancy increase due to light scatter by the model membranes. In the absence of antibody (curves 3 and 4), the absorbancy remains at a constant value; in the presence of antibody, however, further absorbancy increases occur but at a much more rapid rate and to a significantly greater extent in the complete (curve 1), as opposed to the incomplete (curve 2), cuvet. The change in the incomplete cuvet can therefore be attributed to alterations in liposomal light scatter due to agglutination by antibody; the change in the complete cuvet reflects this parameter as well as any untrapped glucose contaminating the liposome preparation and, of course, marker released by antibody–complement. Accordingly, at any given time, the difference in absorbancy between curves 1 and 2 (when corrected for the amount of untrapped glucose added with the liposomes) provides a measure of the quantity of glucose released as a consequence of immune damage to the liposomes. For the specific case described in Figure 4, we obtained 62% trapped glucose release after 30-min incubation in the presence of 9.3 μg antibody/ml (note that there is 0% release in the absence of antibody).

It must be noted that, in this experiment, 4 μl of MC liposomes was assayed that contained 368 nmol of trapped glucose equivalent to an absorbancy of 2.30. From the data in Table II, it can be calculated that a similar experiment with SC liposomes (*i.e.*, containing the same amount of marker) would require assaying 2600 μl (*i.e.*, $4 \times 92.1/0.141$); this is obviously impossible in a "1-ml cuvet." Accordingly, the experiment described in Figure 5 was performed with 200 μl of SC liposomes which, upon release of all trapped glucose, should have produced an absorbancy difference between complete and incomplete cuvets of 0.176. Although a difference was observed (Figure 5), this represented at most a 7% release of trapped glucose attributable to immune damage (*i.e.*, after correction for untrapped marker in the SC liposome preparation and glucose release occurring in the absence of antibody). Moreover, this low percentage of complement-dependent release was only observed in the presence of an antibody concentration (62 μg/ml) that was nearly tenfold higher than that previously shown to saturate MC liposomes actively sensitized with 5% Dnp-Cap-PE (see Table I in Six *et al.*, 1973) (although not shown in Figure 5, there was no significant absorbancy difference between complete and incomplete cuvets containing 9.3 μg of antibody/ml).

Figure 5 also illustrates another disadvantage of the spectrophotometric assay with SC liposomes as a consequence of the need to employ such high concentrations of antibody. Thus, as indicated by the incomplete cuvets (open circles), agglutination of these model membranes produced absorbancy changes that were much greater than those obtained with MC liposomes in the presence of a lower concentration of antibody (see curve 2 in Figure 4). Therefore, unlike the situation with MC liposomes, calculation of the per cent glucose released from SC liposomes must unfortunately rely on a small difference between large numbers (*i.e.*, the absorbancy of incomplete *vs.* complete cuvets).

To circumvent these limitations of the spectrophotometric assay, we attempted the following (results not presented). SC liposomes were prepared that had been actively sensitized with either 0.1, 0.5, or 1% antigen instead of 5% Dnp-Cap-PE as in the preceding experiments. This was predicated on the assumption that antibody produced such pronounced increases in light scatter because the transformation of MC into SC model membranes is accompanied by an enormous rise in the surface:volume ratio of liposomes rendering more antigen accessible to antibody. This modification did indeed reduce (but not eliminate) the extent of light scattering changes but we were unable to detect any complement-dependent release of glucose when different amounts of these liposomes were assayed in the presence of varying concentrations of antibody.[3] Similar negative results were obtained when different amounts of guinea pig serum were added to the cuvets; however, this variable could not be tested over a wide range because, as indicated in Figures 4 and 5, the complement source *per se* possessed an appreciable absorbance at 340 nm (A = 0.4 for an eightfold dilution of guinea pig serum).

[3] A few unsuccessful experiments were also performed with a low affinity rabbit IgG anti-dinitrophenyl antibody (K_0 for ε-Dnp-lysine of 10^5 l. mol^{-1}) substituted for the high affinity antibody. Unfortunately, we did not have available sufficient quantities of the rabbit IgM antibody of comparable low affinity that was used in a previous investigation (Six *et al.*, 1973); this immunoglobulin may have been more appropriate because complement dependent glucose release from MC liposomes requires significantly less IgM, as opposed to IgG, antibody and low concentrations of IgM generally produce little increase in liposomal light scatter.

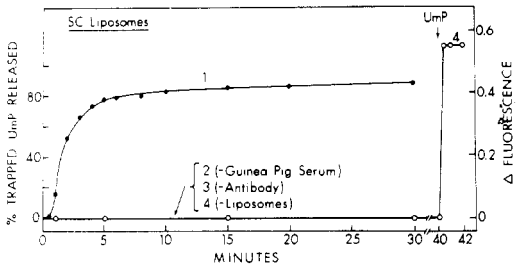

FIGURE 6: Application of fluorometric assay to SC liposomes; kinetics of UmP release. SC liposomes were prepared in a swelling solution that contained 100 mM UmP. The fluorometer tube (curve 1) initially contained 846 μl of complement buffer, 125 μl of guinea pig serum, 5 μl (30 μg) of antibody, and 4 μl (20 μg) of alkaline phosphatase; the reaction was started with 20 μl of SC liposomes. In control tubes, the following substitutions were made: 125 μl of complement buffer for guinea pig serum (curve 2), 5 μl of Tris-saline for antibody (curve 3), and 20 μl of Tris-saline for liposomes (curve 4). For curves 1, 2, and 3, the per cent trapped UmP released (left ordinate) was determined as described in the Experimental Section from the increase in fluorescent readings (right ordinate) at various times after liposome addition. In the case of curve 4, 1 nmol of UmP (in 5 μl of Tris-saline) was added at 40 min that, in the absence of guinea pig serum, would give a reading of 1; thus, under the present experimental conditions, the guinea pig serum quenched fluorescence approximately 44%. See text for further discussion.

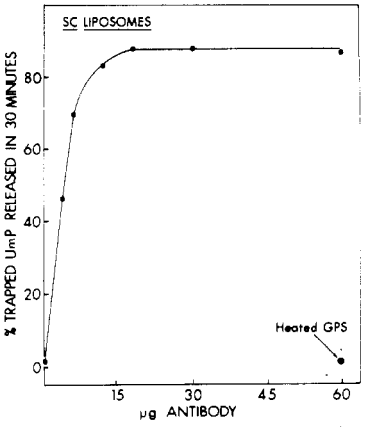

FIGURE 7: Effect of antibody concentration on UmP release from SC liposomes. Procedure identical with that described in the legend to Figure 6 except that varying amounts of antibody (indicated on the abscissa) were added. The control tube contained 125 μl of heated (30 min at 56°) instead of native guinea pig serum (GPS).

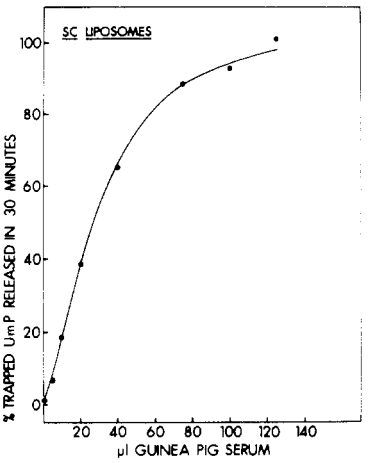

FIGURE 8: Effect of guinea pig serum concentration on UmP release from SC liposomes. Procedure identical with that described in the legend to Figure 6 except that varying amounts of guinea pig serum (indicated on the abscissa) were added.

Application of the Fluorometric Assay. In striking contrast to the spectrophotometric procedure, the fluorometric assay provided conclusive evidence that SC liposomes are susceptible to the action of antibody-complement as illustrated by the time course of UmP release (Figure 6, curve 1) in the presence of both immunologic reagents. No marker was released when either complement source or antibody was omitted (Figure 6, curves 2 and 3), or when the SC liposomes were incubated with high concentrations of antibody and guinea pig serum whose hemolytic complement activity had been destroyed by heating at 56° for 30 min (Figure 7). Another control experiment (Figure 6, curve 4) demonstrates that fluorescence increased much faster upon the addition of free UmP, *i.e.*, marker not trapped in the liposomes. This indicates that, under the conditions of assay, the rate-limiting step is release of marker as a consequence of immune damage to the SC liposomes and not the ability of alkaline phosphatase to hydrolyze UmP. In connection with Figure 6, we need also mention that the control curves 2, 3, and 4 were superimposable (hence not all experimental points have been included) and that the amount of antibody employed (30 μg) did not cause agglutination of the volume of SC liposomes added (20 μl) as indicated by visible examination or the absence of any change in fluorescent readings (curve 2).

Figures 7 and 8 document in greater detail the dependence of marker release on antibody and guinea pig serum with particular reference to the following points. Half-maximal and maximal UmP release from SC liposomes requires approximately 3.8 and 18 μg of antibody/ml, respectively (Figure 7), whereas half-maximal and maximal glucose release from MC liposomes of identical composition have been shown previously to occur in the presence of 1.1 and 8 μg of antibody/ml, respectively (*cf.* Table I in Six *et al.*, 1973). In spectrophotometric assays with MC liposomes, we usually add 35–80 nmol of liposomal phospholipid in contrast to the fluorometric assays with SC liposomes which are performed with 10–15 nmol of liposomal phospholipid. The fact that lower concentrations of antibody suffice to sensitize MC liposomes can therefore also be attributed to the marked increase in total surface area attendant upon their conversion to SC liposomes (see above). Thus, while more antigen is available for combination with antibody in SC liposomes, the probability that two immune complexes are formed in close proximity on the liposomal surface is diminished; previous experiments (Six *et al.*, 1973) have demonstrated that "neighboring" antigen–antibody complexes are necessary to initiate complement-dependent damage of liposomes by IgG immunoglobulins (*cf.* footnote 3). Similarly, comparison of Figure 2 with Figure 8 reveals that in the presence of excess antibody, 4.5 μl of guinea pig serum was sufficient to produce half-maximal marker release (glucose or UmP) from MC liposomes, whereas 27 μl was necessary in the case of SC liposomes.

Despite these differences in sensitivity, one aspect of the behavior of SC liposomes was especially gratifying in view of the circumstances that prompted this investigation (see introduction). Namely, SC liposomes release essentially all of their trapped marker (range 90–100%; Figures 6–8) as a consequence of immune damage. We believe that our failure to consistently obtain complete release of UmP is due to the fact that 125 μl of guinea pig serum is the maximum amount that can be

tolerated in the fluorometric assay (*i.e.*, for which accurate corrections due to quenching can be applied); as shown in Figure 8, this volume does not provide complement in excess.

Discussion

For the purpose of detecting alterations in liposomal permeability, the fluorometric and spectrophotometric assays possess at least two desirable features in common. These are consequences of the fact that both procedures employ (as trapped markers) compounds which, by enzymatic reactions, give rise to products whose concentration can be easily determined. First, the enzymatic reactions can only occur after the liposomal bilayers are no longer able to function as a permeability barrier; this obviates the need for an additional step (such as dialysis or Sephadex chromatography) to separate the released marker from that retained by the liposomes. Second, the K_m and V_{max} of the auxiliary enzymes (*i.e.*, hexokinase and glucose-6-phosphate dehydrogenase, or alkaline phosphatase) are sufficiently low and high, respectively, so that the kinetics of permeability alterations can be followed. The obvious difference between the fluorometric and spectrophotometric assays is the increased sensitivity of the former which has enabled us to demonstrate that SC liposomes undergo immune damage. We should also emphasize that numerous assays can be designed on these principles utilizing substrates other than glucose or UmP, and that these procedures can be employed to investigate a variety of membrane-associated phenomena other than the action of "lytic" agents such as antibody-complement. For example, Haywood (1974) has recently demonstrated the interaction of Sendai virus with liposomes; the sensitive fluorometric assay may well be employed to determine whether these model membranes undergo fusion in the presence of the virus under conditions that maintain the integrity of the liposomal bilayers.

For the purpose of examining the mechanism of immune damage, SC liposomes have several advantages over MC liposomes which are a heterogeneous population of vesicles. First, SC liposomes more nearly resemble a suspension of erythrocytes in that, under optimal conditions, complete release of marker (equivalent to complete hemolysis) can be obtained; second, the precise dimensions of SC liposomes and their concentration can be determined. Thus, Johnson (1973) has calculated that there are approximately 7500 phospholipid molecules per bilayer in SC liposomes which have a contained volume identical with the SC liposomes employed in this investigation. The experiment described in Figure 8 was performed with 13.4 nmol of liposomal phospholipid which corresponds to roughly 10^{12} SC liposomes/ml. With such quantitative information, it should be possible to compare the response of SC liposomes and erythrocytes in several additional ways: for example by determining the efficiency of immune lysis and the stoichiometry of complement component binding in the two systems (*cf.* Mayer, 1961; Kolb *et al.*, 1972). These same advantages make SC liposomes appropriate for examining the molecular basis of complement action by various physical techniques, *e.g.*, electron spin and nuclear magnetic resonance spectroscopy (see introduction). However, on the basis of this study, it should be stressed that such experiments are only valid if performed under conditions (specifically, relative concentrations of liposomes and antibody-complement) which can be shown to promote marker release.

Finally, reference must be made to the recent and significant investigations indicating that the membrane "attack mechanism" of the complement system may involve a complex containing C5b, C6, C7, C8, and C9 (*i.e.*, the last five components) in molar ratios of 1:1:1:1:6, respectively. This proposal originated from studies of the binding of highly purified and radioactive human complement components to the sheep erythrocyte surface (Kolb *et al.*, 1972); it has subsequently received additional confirmation from the finding that these components can interact to form this decamolecular complex (although hemolytically inactive) in solution (Kolb *et al.*, 1973a,b). Obviously, it would be of considerable interest to determine if immune damage to SC liposomes by purified complement components involves a complex of similar stoichiometry because its size (molecular weight 1×10^6) should render it visible by electron microscopy on the liposomal surface. Electron microscopic examination of SC liposomes may also settle the remaining controversy concerning the nature of the lesions (approximately 100–120 Å in diameter) originally detected on the membranes of complement-lysed sheep erythrocytes by negative staining (reviewed in Humphrey and Dourmashkin, 1969) and afterwards in MC liposome preparations by some investigators (Hesketh *et al.*, 1971; Lachmann *et al.*, 1973). However, Polley *et al.* (1971) demonstrated that these lesions were already apparent on the sheep erythrocyte surface after the reaction involving C5 and thus suggested that they may not correspond to functional holes; this conclusion has since been supported by the freeze-etch studies of Iles *et al.* (1973) showing that these lesions do not pass through the erythrocyte membrane (*cf.* also Tillack and Kinsky, 1973). Moreover, other laboratories (Knudson *et al.*, 1971; Kataoka *et al.*, 1973) could rarely, if at all, detect these lesions in MC liposomes after treatment with antibody-complement.

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Transcriptional Control of *in Vitro* $tRNA^{Tyr}$ Synthesis†

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ABSTRACT: The *in vitro* transcription by purified *Escherichia coli* RNA polymerase of the $tRNA^{Tyr}_I$ gene carried by $\phi 80psu^+_3(0)$ phage DNA was studied as a function of transcription factors, ionic strength, and temperature. The synthesis of the $tRNA^{Tyr}$ was determined by competition with the hybridization of *E. coli* [^{32}P]tRNA on the $\phi 80psu^+_3$ (0) separated L-strand DNA. While little tRNA-like material was produced by the purified core polymerase, the σ factor promoted efficient transcription along the $tRNA^{Tyr}_I$ gene. The transcription was found to be salt sensitive, little tRNA synthesis occurring at high ionic strength. Addition of ρ factor enhanced the percentage of tRNA among the *in vitro* transcripts, probably by causing termination of RNA synthesis outside the $tRNA^{Tyr}_I$ gene. Maximal production of tRNA was obtained when transcription was performed by core polymerase + σ in the presence of ρ factor and about 0.05 M KCl. Initiation of $tRNA^{Tyr}_I$ transcription was shown to require a thermal activation, at 28° less tRNA being produced than at 38°. The presence of the protein elongation factors TuTs could not substitute for the thermal activation step.

Transducing bacteriophages carrying genes from bacterial origin have been used in order to study individual transcription units and their regulatory properties (Zubay *et al.,* 1970; Greenblatt and Schleif, 1971; Pannekoek and Pouwels, 1973). The regulation of biosyntheis of tRNA as well as of rRNA (stable RNAs) presents a problem of special interest. Though coded by a minute fraction of the bacterial chromosome (Yankofsky and Spiegelman, 1962; Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962), they constitute a large proportion of all the cellular RNA. The bacterial transcription mechanism must thus be able to discriminate among RNA promoters in favor of the stable RNA species.

We focused our attention on the su_3 gene, the structural gene for *E. coli* tyrosine $tRNA_1$ carried by the $\phi 80psu^+_3(0)$ phage (Andoh and Ozeki, 1968). In order to study the mechanism of tRNA biosynthesis, we transcribed $\phi 80psu^+_3(0)$ DNA by purified *E. coli* RNA polymerase. We have demonstrated (Daniel *et al.,* 1970; Littauer *et al.,* 1971) that the $\phi 80psu^+_3(0)$ DNA can serve as a template for *in vitro* transcription, leading to the synthesis of tRNA like molecules larger in size than 4 S (pre-tRNA). Several other laboratories have also studied $\phi 80psu^+_3$ DNA transcription *in vitro* (Ikeda, 1971; Zubay *et al.,* 1971). Using S_{30} preparations, Zubay *et al.* (1971) reported the successful synthesis of a biologically active $tRNA_{su^+3}$; however, due to the use of crude extracts, the mechanism of tRNA transcription was not investigated. In the present report we have studied the transcription of the transduced su_3 gene by purified *E. coli* RNA polymerase with respect to the effects of transcription factors, ionic strength, and temperature.

Experimental Section

Materials. Nucleoside triphosphates and ^{3}H-nucleoside triphosphates were obtained from Schwarz Bioresearch, Inc. Pancreatic ribonuclease (five times crystallized, A grade) was purchased from Calbiochem. DNase I, electrophoretically purified, was obtained from Worthington. Phage $\phi 80psu^+_3(0)$ was received from Dr. H. Ozeki, and grown on *E. coli* CA 274 (Hfr C Lac^-125_{Amber} $Trp^-{}_{Amber}su^-$) in λ broth agar (Matsushiro *et al.,* 1964). In this phage, transcription of the $tRNA^{Tyr}$gene is from the light (L) DNA strand (Daniel *et al.,* 1970).

Methods. $\phi 80psu^+_3(0)$ DNA preparation, DNA strand separation, and DNA–RNA hybridization were performed as previously described (Daniel *et al.,* 1970). [^{32}P]tRNA was prepared from *E. coli* B cells grown on Tris-glucose medium (Daniel *et al.,* 1969).

DNA-dependent RNA polymerase was purified from *E. coli* MRE-600 cells by the procedure of Chamberlin and Berg (1962). The enzyme preparation was then subjected to low salt glycerol gradient centrifugation (Burgess, 1969) and the core enzyme was separated from the σ subunit by phosphocellulose chromatography (Berg *et al.,* 1971). Termination factor ρ was prepared from *E. coli* MRE-600 cells by the procedure of Roberts (1969); it showed only one predominant band when exam-

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