for about 60 percent of the label incorporated into the ribosomes. Although washing the isolated chloroplasts resulted in about a 40-percent loss of activity, the washing procedure was routinely carried out in subsequent experiments, as it revealed more distinctly the position occupied by heavy ribosomes in sucrose density gradients.

The OD profile of the polyribosome region was shifted to the monosome region by pancreatic ribonuclease (Fig. 2). This treatment also shifted much of the incorporated radioactivity from the polyribosome to the monosome region (Fig. 3), although there appears to be a small amount of heavier material that is less sensitive to ribonuclease.

With the evidence that polyribosomes could be detected by use of washed chloroplasts, the question arose whether the polyribosomes could be released from washed chloroplasts and fractionated on sucrose density gradients before testing the fractions for their capacity to incorporate radioactivity into protein. It was not possible to release sufficient ribosomes for OD analysis by suspending washed chloroplasts at 0°C in a buffered medium of low molarity, according to the method that Boardman *et al.* (*3*) used with unwashed chloroplasts. However, addition of the mixture of combined reagents to the washed chloroplasts at 0°C caused release of ribosomes, generally to about one-half the extent of the ribosomes released during a 1- to 5-minute incubation at 28°C for protein synthesis. By use of combined reagents at 0°C, sufficient ribosomes were obtained in a 17,000*g* supernatant to permit their fractionation by sucrose density gradient centrifugation prior to the incubation of each fraction with reagents necessary for protein synthesis. As shown in Fig. 4, the shape of the absorbancy profile of the ribosomes obtained in this manner is similar to that obtained in previous experiments in which labeled ribosomes were released from washed chloroplasts into the 17,000*g* supernatant as protein synthesis occurred. Figure 4 also shows that more of the protein-synthesizing activity was associated with the polyribosome region than with the monosome region.

This result may be compared with results obtained when washed chloroplasts were first made to incorporate labeled amino acids, and the resulting labeled supernatant was fractionated by sucrose density sedimentation. The specific radioactivity of the polyribosomes was only twice that of monosomes (Fig. 1). Apparently, most of the monosomes carry nascent, or completed, polypeptide chains, and we assume that these monosomes arise by breakdown of the polyribosomes during protein synthesis. Longer incubation periods, as well as mild treatment with ribonuclease, results in the conversion of both radioactivity and OD, originally associated with polyribosomes, to monosome material.

The polyribosome profile of tobacco chloroplasts consists of a much smaller proportion of larger ribosome aggregates, in comparison with monosomes, than those encountered in other plant tissues (*5*) and other organisms (*6*). It can be questioned, therefore, whether the low yields of larger aggregates from the chloroplasts arose from nuclease action on the polyribosomes during the extraction procedure. Boardman *et al.* (*3*) examined mixtures of reticulocyte polyribosomes and tobacco chloroplast supernatants in the analytical centrifuge and concluded that chloroplast supernatants contain little active nucleases. Our chloroplast supernatants were also incubated with reticulocyte polyribosomes (*7*) at 0°C for 15 minutes before resolving the mixture by sucrose density gradient centrifugation. The several humps of the reticulocyte polyribosomes appeared undegraded. Thus, it seems unlikely that the chloroplast polyribosome profile had been altered by nucleases.

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Antibodies to Rabbit Cytochrome c Arising in Rabbits

Abstract. *Antibodies reactive with rabbit cytochrome* c *have been observed in rabbits immunized with several heterologous cytochromes. Such antibodies have also been observed in rabbits immunized with rabbit cytochrome* c *conjugated to bovine gamma globulin. The serum of a rabbit immunized with human cytochrome* c *reacted with the cytochrome* c *of the same rabbit.*

The elicitation of autoantibodies by crude tissue preparations, either by autoimmunization or by heteroimmunization, has been amply described (*1*). Autoantibodies have also been observed after autoimmunization or heteroimmunization with purified extracellular or cytoplasmic proteins such as thyroglobulin (*2*), γ-globulin (*3*), and adenylate kinase (*4*). We describe here the regular appearance of antibody reactive with a mitochondrial protein of the immunized species. Rabbits immunized with a variety of cytochromes *c* from other species produced antibodies to rabbit cytochrome *c* prepared from a pool of several hundred rabbit hearts. Moreover, serum of a rabbit immunized with human cytochrome *c* reacted with cytochrome *c* subsequently isolated from the same rabbit. We have also observed the formation of antibody to cytochrome *c* in rabbits immunized with rabbit cytochrome *c* coupled covalently to acetylated bovine γ-globulin.

White New Zealand rabbits were immunized by either of two methods (*5*). The tuna, turkey, and rabbit cytochromes *c* were injected as conjugates to acetylated bovine γ-globulin while the human and horse proteins were injected as the free native proteins (*5*). Methods used for conjugation and for quantitative estimation of specific antibody have been described (*5*); antibody was estimated by a modification of the Farr technique with the use of I^{125}-labeled cytochrome *c*, by precipitin analysis and by complement fixation.

Cross reactions of antibodies elicited by a particular cytochrome *c*, with the cytochromes *c* of other species, were also investigated by measuring the competition between the homologous I^{125}-labeled protein and the heterologous unlabeled proteins for binding to antibody. On the basis of initial experiments

enough labeled cytochrome *c* was used so that the combining sites of the antibodies were nearly saturated; thus, particular populations of antibody molecules were not selectively utilized.

Ratios of responders to the total number of rabbits immunized with horse, human, tuna, turkey, and rabbit cytochromes *c* were 2:5, 7:8, 3:5, 3:3, and 2:5, respectively.

Antiserums to the human and horse cytochromes *c* precipitated the antigen, fixed complement in the presence of homologous cytochrome *c*, and also bound I^{125}-labeled antigen, as detected by precipitation of the antigen-antibody complexes in the presence of 15 percent Na_2SO_4. The antiserums to tuna, turkey and rabbit cytochromes did not form precipitates but bound the labeled homologous antigen. The antiserums to tuna and turkey cytochromes failed to fix complement; the antiserum to rabbit cytochrome was not tested. All immune serums agglutinated tanned erythrocytes coated with homologous antigen in titers greater than 160; corresponding values for normal serums were less than 2. The antiserums used bound the following amounts of homologous, I^{125}-cytochrome *c* per milliliter of serum at the highest concentrations of antigen tested; antiserums to horse (rabbits 6B and 7B), 31 and 27 μg; antiserum to human (rabbit 76B), 20 μg; antiserum to tuna (pool from rabbits F2 and F5), 56 μg; antiserum to turkey (rabbit TH4), 140 μg; antiserums to rabbit (rabbits R1 and R7), 1.7 and 0.7 μg.

In the complement fixation reaction of rabbit cytochrome *c* with two serums prepared against horse cytochrome *c* and two serums prepared against human cytochrome *c* (Fig. 1), the reactions with rabbit cytochrome *c* fixed 15 to 30 percent as much complement as those with the homologous cytochromes *c*.

The maximum amounts of antibody nitrogen, per milliliter of serum, precipitable by rabbit cytochrome *c* from the two antiserums to human cytochrome *c* (from rabbits 74B and 76B), were 30 and 15 μg, respectively. These values represented 30 and 25 percent of the maximum amounts of nitrogen precipitated by the human protein. Thus, a similar extent of cross-reaction is indicated by complement fixation and precipitation. The two antiserums to horse cytochrome *c* were not sufficiently strong for quantitative precipitin analysis of the cross-reaction with rabbit cytochrome *c*. In agar-diffusion experiments, however, faint lines were seen when rabbit cytochrome *c* was allowed to diffuse against the antiserum to horse cytochrome. With both antiserums, the horse-protein line formed a spur over the rabbit-protein line in a typical reaction of partial identity.

Figure 2 shows the binding of I^{125}-labeled rabbit cytochrome *c* by rabbit antiserums specific for various "mammalian-type" cytochromes *c*, including that of rabbit. Corrected for dilution and for the small amount of binding by the normal serum, the amounts of rabbit cytochrome *c* bound by 1 ml of the various antiserums to cytochrome *c* at the highest points on the curves are: antiserum to human (rabbit H4), 22 μg; pooled antiserum to tuna (rabbits F2 and F5), 18 μg; antiserum to turkey (rabbit TH4), 43 μg; antiserum to horse (rabbit 7B3), 18 μg; antiserum to rabbit (rabbit R1), 1.7 μg. A number of other active serums, not represented in Fig. 2, and prepared against tuna, turkey, human or horse cytochromes *c*, were also tested; in every case a significant amount of rabbit cytochrome *c* was bound. The largest amounts of rabbit cytochrome *c* bound (Fig. 2) are not maximum, since binding continues to increase with the concentration of added antigen; however, with very high concentrations of antigen the percentage of the available antigen bound became small and therefore could not be determined accurately.

Binding of rabbit cytochrome *c* by heterologous antiserums was also demonstrated in competition experiments. The extents of binding of I^{125}-labeled horse, human, tuna or turkey cytochromes *c* to their respective homologous antibodies decreased in the presence of unlabeled rabbit cytochrome *c*. The antiserums were diluted before use with normal rabbit serum in ratios ranging from 1:7 to 1:72. The amount of I^{125}-labeled cytochrome *c* present was nearly sufficient to saturate the combining sites of the antibody. The maximum displacements observed in the presence of increasing amounts of unlabeled rabbit cytochrome *c* were: 62 percent (3 μg horse I^{125}-cytochrome *c* reacting with antiserum to horse cytochrome *c*); 52 percent (0.75 μg human I^{125}-cytochrome *c* reacting with anti-

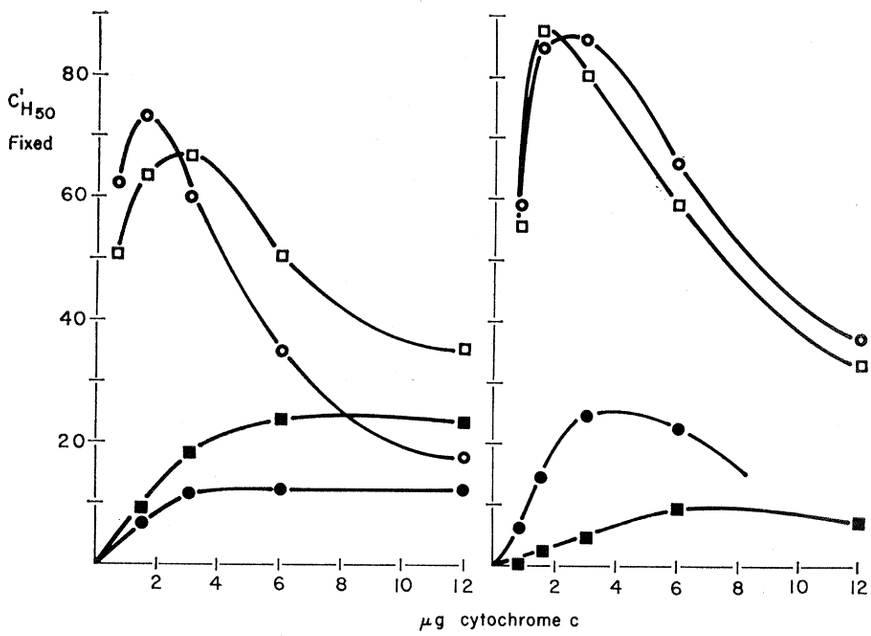

Fig. 1. Complement fixation curves comparing reactions of horse and rabbit cytochromes *c* with rabbit antiserums to horse cytochrome *c* (left) and human and rabbit cytochromes *c* with rabbit antiserums to human cytochromes *c* (right). The extent of complement fixation was determined according to Mayer *et al.* (*8*) except that 1-ml rather than 10-ml quantities were used, the concentration of all reactants remaining the same. The ordinate represents units of complement based on a 10-ml reaction mixture. Upper curves in both figures (○, □) refer to the homologous cytochromes (human and horse) while the lower curves (●, ■) are the rabbit cytochrome *c* cross-reactions. On the left, (○, ●) refer to serum 6B and (□, ■) to serum 7B, both rabbit antiserums to horse cytochrome *c* and both used in 1/16 dilution. On the right (○, ●) refers to serum 76B and (□, ■) refers to serum 74B, both rabbit antiserums to human cytochrome *c* and both used in 1/50 dilution. All dilutions are in the standard buffer used for complement fixation experiments.

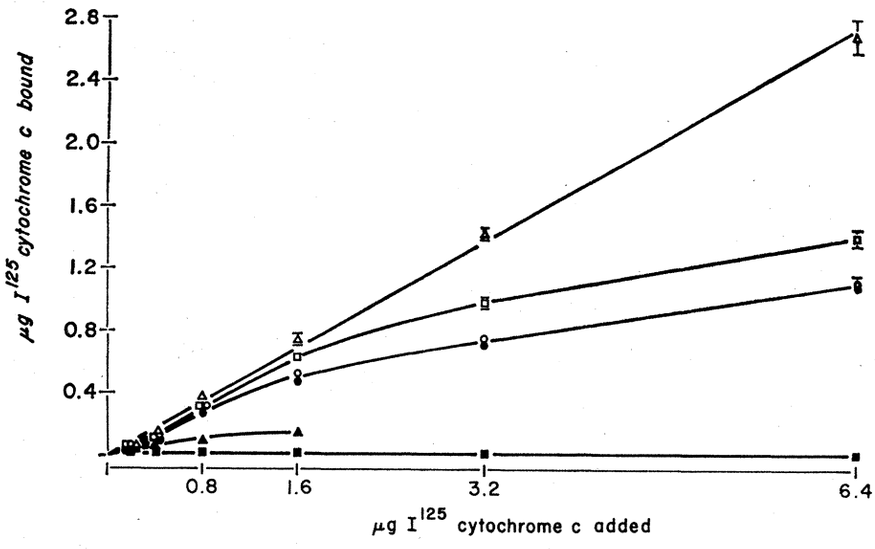

Fig. 2. Binding of rabbit I^{125}-cytochrome *c* by rabbit antiserum against turkey (△), human (□), horse (○), tuna (●) and rabbit (▲) cytochromes and by the pooled serums of nonimmunized rabbits (■). Each antiserum was diluted with four parts of the pooled normal serums. Binding measurements were carried out by a modification of Farr's method (*5*); 0.3 ml of the diluted antiserum was used in each test. Experiments were carried out in duplicate; where the range of the two measurements exceeded the size of the symbol, this is indicated by a vertical bar.

serum to human cytochrome *c*); 31 and 55 percent (8 μg tuna I^{125}-cytochrome *c* reacting with each of two antiserums to tuna cytochrome *c*); 89 percent (10 μg turkey I^{125}-cytochrome *c* reacting with antiserum to turkey cytochrome *c*). The ratios of rabbit cytochrome to labeled antigen required for maximum displacement varied between 20 : 1 and 50 : 1. Thus, rabbit cytochrome *c* can react with rabbit antiserums directed against heterologous cytochromes *c*.

Evidence for the presence of antibodies against rabbit cytochrome *c*, in rabbits immunized with rabbit cytochrome *c* coupled to bovine γ-globulin, was obtained by the passive hemagglutination test (*6*), in which tanned sheep red blood cells coated with rabbit cytochrome *c* were used. Of the two rabbits that responded to immunization, the serum of rabbit R1 gave a titer of 640 and that of rabbit R7 a titer of 1280. The titers of several normal rabbit serums were less than 2. The results confirm those of the direct-binding experiments.

A rabbit (H7), immunized with human cytochrome *c*, was killed, after it had been bled repeatedly for serum. Cytochrome *c* was isolated from its muscle tissue and labeled with I^{125}. The binding capacity of the antiserum of this rabbit for its own cytochrome *c*, measured by the modified Farr technique and calculated for the undiluted antiserum, was 55 μg of cytochrome *c* per milliliter. This value is corrected for the small amount of binding shown by pooled normal rabbit serum (< 1 μg/ml). The antiserum was diluted with four parts of normal serum for the tests.

Our observations show that antibodies arising in rabbits during immunization with cytochromes *c* prepared from horse, human, tuna, or turkey hearts cross-react to considerable extents with rabbit cytochrome *c*. Moreover, it is possible to elicit small amounts of antibody to rabbit cytochrome *c* in rabbits by the injection of rabbit cytochrome *c* conjugated to acetylated bovine γ-globulin. (The conjugated preparations also contain some free cytochrome *c*.) We have not been able to detect any antibodies in rabbits injected with native rabbit cytochrome *c*.

Our results raise the question whether rabbits are immunologically tolerant to rabbit cytochrome *c*, or whether the phenomenon of tolerance is unrelated to our observations. For those of their own tissues, such as lens, to which animals are not tolerant, immunization with protein preparations from such tissues leads to an excellent antibody response. The relatively poor response to the conjugated homologous cytochrome *c*, as compared to the strong responses obtained by immunization with conjugated heterologous cytochromes *c*, is therefore suggestive of a tolerant state. However, the number of animals immunized is insufficient to permit a definite conclusion with regard to the immunogenicity of rabbit cytochrome *c* in rabbits. If tolerance is the normal state, one might expect that it could be broken by substances related to, but differing from the native rabbit protein, possibly including rabbit cytochrome *c* conjugated to bovine protein. Such observations have indeed been reported with regard to the termination of the tolerant state to a protein antigen by administration of derivatives of the protein or of related proteins from other species (*7*). Thus, the present instance is possibly an example of a similar event occurring on a background of natural tolerance. In such a case the study of the immunological behavior of a large series of cytochromes *c* of known primary structure may prove useful in probing the phenomena underlying the mechanism of immunological tolerance.

It seems likely that the sort of immunological behavior exhibited by cytochrome *c* in rabbits will prove to be the case for many intracellular proteins. The recent elicitation of antibodies in rabbits directed against rabbit adenylate kinase by immunization with the guinea pig enzyme (*4*) provides such an example with a cytoplasmic enzyme. Our work extends this finding to a mitochondrial protein.

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