MOLECULAR ASSOCIATION OF HEMOCYANIN PRODUCED BY X-RAYS AS OBSERVED IN THE ULTRACENTRIFUGE

By EDWARD G. PICKELS and RUBERT S. ANDERSON

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York, the Department of Physiology, School of Medicine, University of Maryland, Baltimore, and the Memorial Hospital, New York)

(Received for publication, June 20, 1946)

Numerous examples have been cited in the literature of the changes produced through the action of ionizing radiations and particles on proteins (1–3). The effect has been detected and measured in several different ways. In the case of viruses (4, 5), bacteriophage (6, 7), and enzymes (8–11), a loss of activity occurs. With other proteins, changes in the spectral absorption of irradiated solutions have been noted (1, 12), and with very large amounts of either ultraviolet light (13) or x-rays (2), proteins have been rendered sufficiently insoluble (denatured) to exhibit visible flocculation. Serum albumin has been examined in the ultracentrifuge after subjection to ultraviolet light (14, 15), α particles (15), and small doses (29,000 roentgens) of soft x-rays (12). No effect from the x-rays was observed but limited amounts of the other two radiations were found to produce an inhomogeneous protein, the altered material sedimenting at both higher and lower rates without the formation of resolvable boundaries. On the other hand, the large hemocyanin molecules of *Helix pomatia* have been observed, through ultracentrifugation, to be split into halves by the action of either ultraviolet light or α particles (15, 16).

In the study herewith described, an ultracentrifugal examination of hemocyanin (*Limulus polyphemus*) irradiated with certain dosages of x-rays has disclosed a partial transformation of the protein from its originally monodisperse form into several discrete, more rapidly sedimenting components which are relatively stable and homogeneous. This indicates an association of the hemocyanin molecules with the formation of several different simple combinations. The amount of material which is altered varies, reproducibly, according to the amount of radiation, the concentration, the amount of extraneous protein present, and other conditions described below.

Materials and Methods

The hemocyanin was concentrated and purified from the blood of *Limulus polyphemus* by filtration and differential centrifugation. Freshly drawn blood was allowed to stand at 4°C. for several days and then filtered. Subsequently it was spun in an angle

centrifuge (17, 18) at 30,600 R.P.M. for 3 hours. The gelatinous pellets of hemocyanin were resuspended to the original volume in 0.17 M saline buffered with phosphate at pH 6.9. The material was again thrown down in the centrifuge and resuspended in a fresh, but smaller, quantity (1/18) of the same buffered salt solution. This concentrated stock material was stored at 4°C. under toluol.

Except where otherwise noted, the stock solution of hemocyanin was always diluted to the required concentration with either the same buffer (salt medium), or with preparations of horse serum, or egg albumin which had been dialyzed against frequent changes of this medium until the pH was the same. The concentrations of the hemocyanin and albumin stock solutions were determined from the areas under their refractive index curves obtained in the ultracentrifuge.

During irradiation the material was held in stoppered (rubber) glass tubes 30 mm. in length, 7 mm. in diameter, and 0.4 mm. in thickness. They were kept at room temperature by a shallow water bath. A constant intensity of x-rays, amounting to 2,250 roentgens per minute, was administered sufficiently long to give doses of 200,000, 500,000, and 600,000 r. Peak voltage was 180,000 volts and the radiation was unfiltered except for the glass of the x-ray tube.

After irradiation the small tubes were stored at 4°C. until ultracentrifugation could be performed. Repeated analyses were made on most preparations in order to observe the effects of storage. All of the more concentrated preparations were diluted with buffered saline to a hemocyanin content of 0.8 per cent for the ultracentrifugation and therefore stored after the first run in this condition at 4°C., except where otherwise indicated. The specimens were kept in tightly stoppered glass tubes and were covered with toluol in most instances.

In some experiments the oxygen content of the hemocyanin was reduced by passing nitrogen through the solution. The gas was introduced through a fine needle and a continuous stream of fine bubbles was allowed to flow for 10 minutes. The glass tube, previously drawn down to a size only slightly larger than the needle, was then sealed off. In other cases, partial evacuation with a water aspirator was employed. The solution was placed in a flask having a side arm which could be sealed off by flaming. Enough water was first added to allow for expected losses as previously determined. The flask was swirled around and the fluid maintained in a thin layer during aspiration to minimize frothing. When the escape of gas from the fluid ceased, the flask was tilted and the material allowed to flow into the side arm.

The sedimentation behavior of the hemocyanin was studied in the air-driven ultracentrifuge of Bauer and Pickels (19, 20), at a speed of 30,600 R.P.M. Photographic measurements were made of the refractive index gradients with a modified form (20, 21) of the Philpot-Svensson method. The green and yellow lines of the mercury arc were used for illumination. Base lines for the photographs were determined through runs made at the same speed with buffered saline only. All photographs were optically enlarged and areas under the traced curves were measured by a planimeter.

EXPERIMENTAL RESULTS

Ultracentrifugal Behavior of Unirradiated Hemocyanin.—From previous studies (Pickels, unpublished) it was known that in fresh untreated *Limulus* blood practically all of the respiratory protein exists in the form of a single ultra-

centrifugal component (molecular weight approximately 3,400,000) of sedimentation constant 60.5S, and that this component could be purified by the procedure already described without serious alteration in the physical properties of the protein. Furthermore, it was known that the purified protein remained relatively stable if properly handled, but could be partially dissociated by a variety of comparatively mild treatments, such as warming or storing in the frozen state. Reassociation would take place when conditions were brought back to normal, but only very slowly, several weeks being required for dilute preparations. It seems probable that the preparations studied by Svedberg and his associates (22, 23) were in a state of incompleted reassociation, since

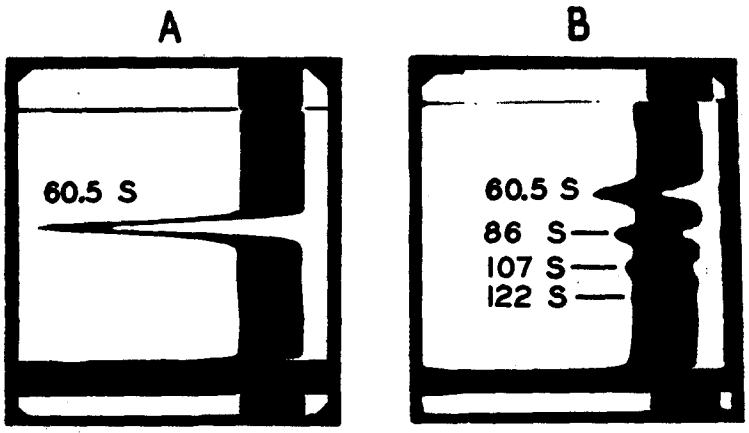

FIG. 1. Refractive index photographs, taken with the ultracentrifuge, of hemocyanin (0.8 per cent) from *Limulus polyphemus*. Photograph A shows normal material in neutral buffer. Photograph B shows new components produced by irradiation with 600,000 r. The base line of photograph A is displaced less than that of photograph B because the picture was taken at reduced speed to permit broadening of the boundary by diffusion.

they observed several components and were led to believe that the one of sedimentation constant 34S was the principal one.

Although only a single component (see photograph A of Fig. 1) was readily discernible in most of the purified "normal" preparations, careful measurements of the refractive index curves commonly indicated the presence of a very small amount of more rapidly sedimenting material (constant of about 86S), the significance of which is discussed later. Apparently the amount of this material gradually increased during storage and was observed to approach 10 per cent of the total concentration after 8 months. It is very probable that the amount also depended on the care taken in handling and preparing the material, especially during its resuspension from the centrifuged pellet. Also, long storage appeared to decrease the concentration of the principal component slightly more than could be accounted for by the increase in the discernible heavy component.

Therefore, for purpose of control, samples of the unirradiated stock solutions were periodically analyzed in the ultracentrifuge and account was taken of the small amount of rapidly settling material in measuring the effect produced by irradiation.

Principal Effect of Irradiation on Hemocyanin in Buffered Saline.—The ultracentrifugal behavior of hemocyanin before and after treatment with x-rays is illustrated by photographs A and B of Fig. 1 and photograph B of Fig. 2. Irradiation caused a reduction in the concentration of the normal component without appreciable alteration of its sedimentation constant. New, relatively homogeneous components appeared, their number (within the limits of ultracentrifugal differentiation) and individual concentrations increasing with the dose. At least three components were well differentiated in several different experiments. Their sedimentation constants were found reproducible to within a very few per cent, with average values of 86S, 107S, and 122S. With the amounts of radiation used (200,000 to 600,000 r), the concentration of each component was always higher than that exhibited by the next component of higher sedimentation rate.

The new components were found to possess a degree of physical stability quite comparable to that of the normal component, as indicated by their ability to withstand a variety of unfavorable treatments. For example, storage for 8 months at 4°C. produced only minor changes (discussed later) in the ultracentrifugal pattern. Also, the new components were able to undergo centrifugal packing without gross loss of solubility, as illustrated by the following experiment. A 0.82 per cent solution was irradiated with 200,000 r and subsequently found to exhibit components 60.5S, 86S, and 107S in the respective concentrations of approximately 0.57, 0.17, and 0.08 per cent. The material was centrifugally packed at 30,600 R.P.M. into a pellet, which was then allowed to redissolve in the ultracentrifuge cell at 4°C. over a 5-day period. A second ultracentrifugation showed the same components to be still sharply defined. In addition, the 122S component could also be differentiated. The respective concentrations were approximately 0.39, 0.16, 0.09, and 0.05 per cent, indicating a significant decrease in the amount of normal protein and a slight increase in the total amount of the heavier soluble material.

Additional evidence of stability was afforded by the comparative ability of the new components to withstand changes in pH sufficient to cause partial dissociation of the normal component. For example, when a 0.8 per cent solution which had been irradiated in the usual way was dialyzed for 6 days against a buffer of pH 7.6, about one-fourth of the normal component was observed to have dissociated into smaller components just as it does with unirradiated material. The components which had been produced by treatment with x-rays were still present and appeared to have suffered only a slight decrease in concentration.

The ability of the new 86S component to withstand heating was comparable to

that shown by the normal material, as illustrated by the following experiment. A 14.9 per cent solution of hemocyanin which had been irradiated with 200,000 r

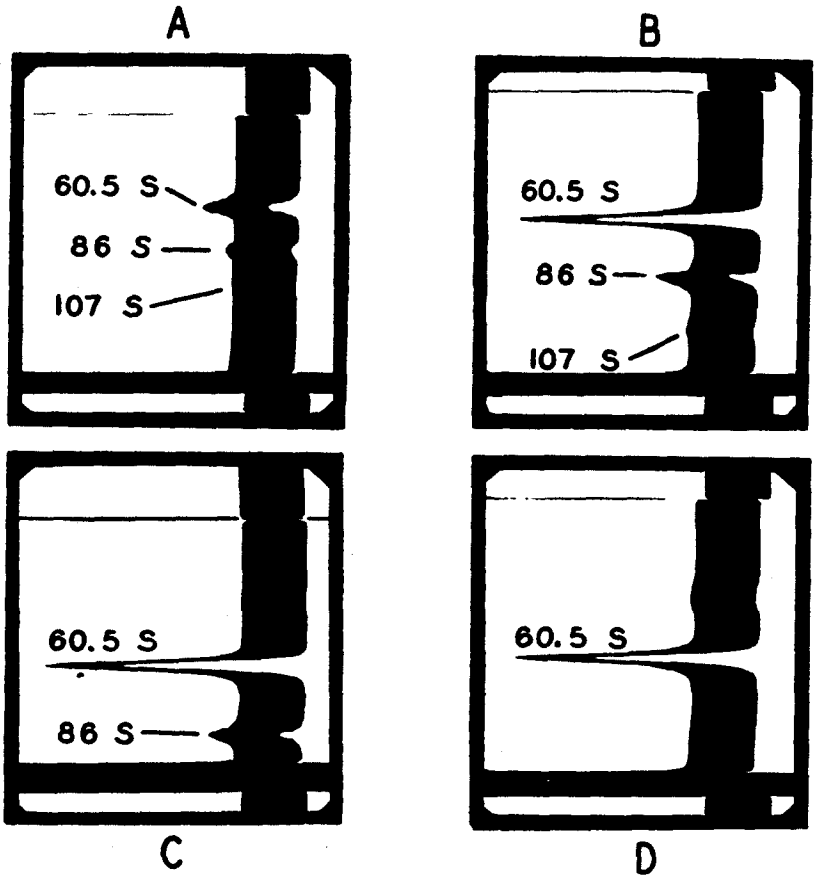

FIG. 2. Ultracentrifuge photographs illustrating the decreasing fraction of the total hemocyanin affected with increasing concentration of the hemocyanin in buffer. The protein concentrations of preparations A, B, C, and D (gelatinous pellet) at the time of irradiation (200,000 r) were respectively 0.2, 0.8, 15, and 45 per cent. C and D were diluted to 0.8 per cent for ultracentrifugation. Effect of dosage is shown by comparison of B with photograph B of Fig. 1. The very small amount of a slower component (34S) exhibited by preparation D (gelatinous pellet) may represent a dissociation of a few hemocyanin molecules by the x-rays. However, slight amounts of dissociation into the 34S component have been observed also with unirradiated material immediately following its resuspension from a centrifuged gelatinous pellet.

showed about 20 per cent of the material to have been transformed into the 86S component. When this solution, diluted 18 times with buffer (or with dialyzed horse serum), was heated at 56°C. for one-half hour, about one-third of the

principal component (60.5S) dissociated into a smaller component (34S), acting very much like unirradiated material in this respect. Although a loss of the 86S component was also noted, almost 50 per cent appeared unchanged. It is of interest here that during 10 days of subsequent storage at 4°C. reassociation took place, almost completely in the case of the principal component, and to about 75 per cent in the case of the 86S component. These observations do not necessarily mean that the particular bond formed between hemocyanin particles by the action of x-rays was itself affected by the heating.

Additional Effects of Irradiation with Buffered Saline as Medium.—The primary effect discussed above related to the appearance of measurable components following irradiation. There were also other more obscure effects relating particularly to a portion of the material which could not be accounted for by the combined concentrations of all the measurable components alone. When solutions of 0.2, 0.8, and 14.4 per cent were examined soon after irradiation with 200,000 r, the unaccounted for portion amounted to about 15 per cent of the starting material. An experiment with the more concentrated of these solutions and a higher dose of 500,000 r showed more than 20 per cent to be unaccounted for by the measurable boundaries. However, in every case the presence of some more rapidly sedimenting material was definitely indicated by a failure of the refractive index curve to coincide with the base line. In some cases there was also suggestive evidence of very small amounts of material sedimenting at rates lower than that of the normal component.

When the preparations just described were allowed to stand at 4°C. for several weeks or months, it was found that the normal component gradually increased in concentration while the other measurable components appeared to remain unaffected or to suffer only very slight changes in concentration. The unaccounted for portion decreased to less than half of what it had been originally. The same phenomenon was observed with a specimen whose oxygen content before irradiation had been reduced by aspiration. It was noted that this recovery of the principal component was most pronounced with preparations which were stored in the concentrated state.

One 0.75 per cent solution was irradiated with 600,000 r and the unaccounted for portion was determined to be 40 per cent of the original material. There was positive evidence in this case (see photograph B, Fig. 1) of material sedimenting considerably more slowly, as well as faster, than the resolvable components.

Variation of Principal Effect with Amount of Irradiation.—Although most of the x-ray exposures were done with 200,000 r, a few experiments were performed with dosages of 500,000 r and 600,000 r. Photograph B of Fig. 1 and photo graph B of Fig. 2 illustrate the variation of effect with dosage. With an 0.8 per cent solution, the proportion of the material affected increased from about 44 per cent to 70 per cent as the dose was increased from 200,000 r to 600,000 r. In a 14 per cent solution it increased from 22 per cent to 42 per cent when the

dose was raised from 200,000 r to 500,000 r. Obviously there was a significant relationship between the amount of irradiation and the size of the effect produced. Whether or not a direct proportionality between dosage and effect exists cannot be decided with certainty from these data, since the exact mechanism whereby the normal material is transformed and hence the correct basis of measurement are not known. However, even when the comparisons are made on a logarithmic scale (dosage *versus* log Co/C), it still appears that the irradiation is slightly less efficient at the higher dosages, though possibly not significantly so.

Variaiton of Principal Effect with Concentration of Hemocyanin.—When buffered saline was used as the suspending medium, the fraction of the material affected during irradiation at 200,000 r decreased as the concentration of the material was increased, as illustrated by Fig. 2. The fraction of material affected amounted to approximately 0.4, 0.3, 0.2, and 0.02 when the concentration of hemocyanin was respectively 0.2, 0.8, 15, and approximately 45 per cent (gelatinous pellet). The absolute change, in terms of actual amount of protein affected, was approximately 0.8 gm. per liter, 2.4 gm. per liter, 30 gm. per liter, and 10 gm. per liter (very approximate) for the respective concentrations cited above. Experiments were done also on dried material and no effect of irradiation was observed. However, this apparent absence of the effect cannot be accepted as conclusive because of experimental difficulties encountered with such material. Desiccated hemocyanin does not redissolve easily and appears to be at least partially denatured.

Influence of Added Egg Albumin.—To determine what influence an extraneous protein of known molecular size would have during irradiation, experiments were performed in which dialyzed egg albumin was incorporated in the medium to the extent of 2.8 per cent. The results are illustrated by the photographs in Fig. 3. Under these conditions two separate solutions of hemocyanin (0.8 per cent) exposed to 200,000 r showed only about one-third as much of the modified *Limulus* protein as observed in the absence of egg albumin. Storage of the irradiated mixture for 40 days produced no appreciable change. A single experiment in which the dose was increased to 600,000 r showed little, if any, additional effect. This particular preparation was subjected to two separate irradiations 24 hours apart. When the hemocyanin concentration was increased to 15 per cent, albumin, still 2.8 per cent, exerted relatively little inhibitory effect (see B, Fig. 3). The modified hemocyanin still amounted to about three-fourths of that observed with simple buffer. This was true for dosages of both 200,000 r and 600,000 r. Hence, although the albumin reduced the proportional effect in the dilute hemocyanin solution from a ratio of 0.3 to 0.1, in the more concentrated solutions the original effect of 0.2 was reduced only to 0.15. Therefore, in the presence of albumin there occurred a reversal of the proportional effect which has been described for simple buffer media; *i.e.*, the effec-

tiveness of the x-rays, on a proportional basis, was now greater with concentrated preparations than with dilute ones.

That egg albumin itself does not modify the ultracentrifugal behavior of hemocyanin, even after several weeks' storage of the mixutre, was shown by a number of preliminary control experiments.

A second, but less well defined, influence of egg albumin was also noted. As already mentioned, all of the starting material could not be accounted for by the resolvable components alone when hemocyanin was irradiated in buffer solution and observed within a few days after the irradiation. On the contrary,

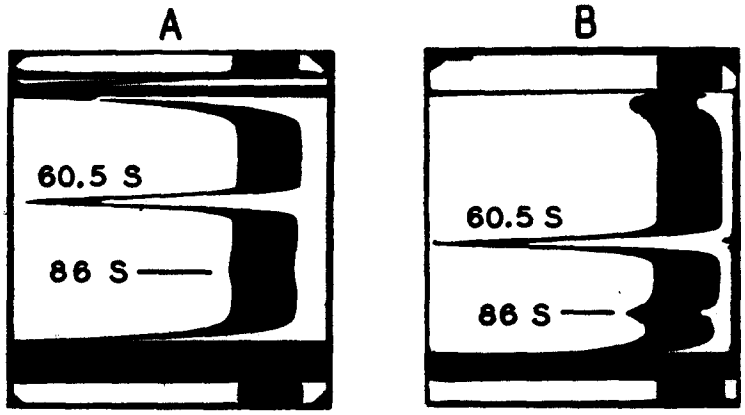

FIG. 3. Photographs showing egg albumin inhibition and the reversal of the proportional effect (*i.e.*, the fraction of the total hemocyanin modified by the radiation) obtained by irradiating (200,000 r) a 0.8 per cent (A) and a 15 per cent (B) hemocyanin solution in the presence of 2.8 per cent egg albumin. B was diluted to 0.8 per cent for the ultracentrifugation. In contrast to the results with simple buffer (see B and C, Fig. 2), the proportional effect was greater in the more concentrated solution. Sedimentation of the albumin can be seen near the meniscus.

measurements of the total area under the refractive index curves of specimens irradiated in the presence of albumin showed, in varying degree, more material sedimenting with the hemocyanin boundaries than could be accounted for by the hemocyanin alone. In dilute specimens irradiated with 600,000 r and in the concentrated samples treated with the smaller dose, the proportional increase was about 10 per cent. It amounted to more than 20 per cent in an experiment with the concentrated material subjected to 600,000 r. This particular preparation (2.8 per cent egg albumin) had been stored for 8 days in the concentrated state before being ultracentrifuged. It was noted that in general a greater proportional increase was observed with specimens which had been stored in the concentrated state after irradiation. No such increase in concentration was

observed when unirradiated hemocyanin was mixed with albumin. Nor was it noticed when unirradiated albumin was added to hemocyanin (0.8 per cent) which had been irradiated in buffer 32 days previously (at a concentration of 15 per cent). Also, the appearance of the components produced by x-ray was not affected in this instance by the addition of albumin, indicating that the inhibitory effect of albumin could be exerted only if the protein were present during the irradiation. Incidentally, a sample of this mixture (irradiated hemocyanin plus unirradiated albumin) was stored for almost 5 months and again tested. The concentration of the normal component was found to have

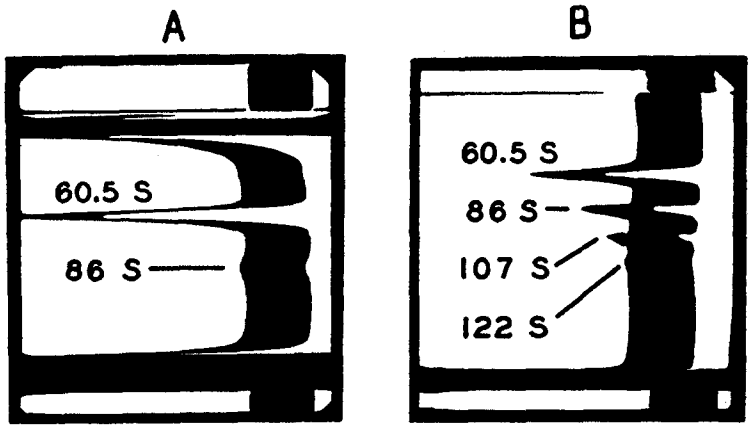

FIG. 4. Photographs depicting the influence of horse serum and of oxygen. A illustrates the inhibiting effect of 33 per cent horse serum present during the irradiation of hemocyanin with 200,000 r (compare with B in Fig. 2). B shows the enhanced production of new components accomplished with 200,000 r when the oxygen tension in the solution was reduced below the atmospheric concentration. (Compare with B in Fig. 2.)

increased by about 20 per cent. It will be recalled that increases of a similar nature were observed with simple buffer media after storage.

Influence of Added Horse Serum.—A few experiments similar to those described above were performed on hemocyanin (0.8 per cent final concentration) mixed with dialyzed normal horse serum. The mixtures contained one-third part by volume of serum and hence were comparable in protein concentration to the solutions containing egg albumin. When present during irradiation, the dialyzed serum greatly inhibited the principal effect of the x-rays. This observation is illustrated by photograph A of Fig. 4. As with albumin, an apparent small increase in the total concentration of hemocyanin was noted. It was still present when examinations were made 5 weeks later. On the other hand, dialyzed serum added to previously irradiated hemocyanin did not

detectably alter the appearance of the new components, whether observed immediately or after 10 days' storage. Apparently the inhibitory effect depended upon the presence of the serum during the irradiation.

Additional experiments performed with undialyzed horse serum were in essential agreement with those described above, although a variable tendency of both irradiated and unirradiated mixtures to precipitate on storage made interpretation of many experiments uncertain. Nevertheless, where mixtures containing either 0.8 per cent or 14 per cent hemocyanin were tested very soon after irradiation, the total effect was found to have been almost completely inhibited by the serum, even with dosages of 500,000 r. The marked inhibition with concentrated material is in contrast to that already described for concentrated hemocyanin irradiated in the presence of albumin, which caused only slight inhibition.

Separate experiments showed that, as with dialyzed serum, there was no inhibition or alteration of the new components when the serum was added after the irradiation of the hemocyanin. Of incidental interest here is the observation that no effect on unirradiated hemocyanin was noted when it was mixed with irradiated serum.

The lessened stability of the mixtures containing undialyzed serum is unexplained, although inconstancy of pH may have been partly responsible. One hemocyanin-serum mixture was tested and found to have a pH of 7.3 as compared with 6.9 in the dialyzed preparations.

The Influence of Oxygen.—As it has been found previously that dissolved oxygen influences the quantitative effect of x-rays on certain compounds (2), including at least one protein (Anderson, unpublished), it was desirable to study its influence during the irradiation of hemocyanin, especially since oxygen readily combines with this protein. Most, but not all, of the oxygen can be removed at room temperature by lowering the oxygen tension through aspiration or flushing with an oxygen-free gas, such as nitrogen (24). When 0.8 or 14.4 per cent solutions of hemocyanin in buffer were so prepared before x-raying, the effect of the irradiation was found to be greatly enhanced, as illustrated in photograph B of Fig. 4. In some cases, the effect was almost doubled. The same treatment applied to unirradiated hemocyanin caused no appreciable change in the ultracentrifugal pattern of the protein.

To determine whether or not the new ultracentrifugal components observed after irradiation of hemocyanin might depend on the production of hydrogen peroxide by the x-rays, an ultracentrifugal examination was made of the unirradiated protein in a buffer solution containing 0.002 M hydrogen peroxide. Theoretically, this was much more peroxide than could have been produced by the x-rays in the experiments described (25). No effect on the protein was observed.

Miscellaneous Observations.—Theoretical concepts evolved from an analysis

of all the collected experimental data suggested the possibility, as explained later, of producing, by heating irradiated hemocyanin at 56°C., a small increase in the amount of material sedimenting at some rate intermediate to 60.5S and 86S. Consequently a careful re-examination was made of the refractive index curves of all experiments involving heating, although unfortunately they were not particularly well designed for testing this point. In no case could a resolvable intermediate boundary be detected. However, the curves for the few ultracentrifugations made soon after heating did indicate a very slight rise in the concentration of intermediate material and an increased spread of the 60.5S and 86S boundaries toward each other near the base line.

A few miscellaneous observations, possibly having no particular significance with respect to the main investigation, were made with normal hemocyanin (2.8 per cent) which had been dissociated into a single component (16S) by suspension in a medium of pH 10. When this solution was irradiated at 200,000 r and diluted to the usual concentration (0.8 per cent) no difference in the behavior of this slowly sedimenting boundary was noted. When the pH was restored to 6.9 by dialysis for 12 days about 25 per cent of the material had reassociated into the 60.5S component. There was no evidence of any component sedimenting at a higher rate.

DISCUSSION

It is well known that radiation of the type used in this study produces its chemical effects through the ionization of molecules exposed to it. Regarding the observed association of hemocyanin particles, the only hypothesis which seems consistent with the experimental data is that such ionization, either of the surrounding water or of the hemocyanin itself, leads to an "activation" or "sensitization" of a portion of the individual hemocyanin particle. Whether only one or both of two particles need be sensitized for them to associate cannot be said with certainty.

The ultracentrifugal boundaries of abnormally high sedimentation rate very probably represent aggregates of 2, 3, and 4 hemocyanin particles respectively. Higher orders would be expected and would account for the rapidly settling material which was not resolved into separate components. The relationship existing between the sedimentation constants of the several components is quite consistent with what is to be expected on the assumption of simple aggregates (26). Such multiple boundaries have been observed in the case of vaccine virus (26), aggregated from unknown causes, and approximately the same ratios of new to normal sedimentation rates were found to exist. It is interesting to note that the sedimentation constant of the x-ray–produced doublets (86S) coincided with that of a small amount of material observed in varying concentration in "normal" hemocyanin which had been purified and concentrated, especially after long storage. This suggests that the aggregation

associated with the new ultracentrifugal components is not unique and may occur under various conditions other than exposure to x-rays.

Although the gross aggregation or precipitation of proteins by very large doses of various radiations is a common observation, ultracentrifugation has given little information about the succession of changes which the proteins undergo before flocculation becomes perceptible. In the case of serum albumin exposed to ultraviolet light, Pedersen (14) and Sanigar, Krejci, and Kraemer (12), and in the case of hemoglobin, Svedberg and Brohult (15), have observed in the ultracentrifuge more rapidly sedimenting material presumably consisting of small aggregates. This material was not resolved into separate boundaries. It seems likely that the association of these ultraviolet–treated proteins did not produce such discrete multiples as were observed with the x-ray–treated hemocyanin, which would not be surprising in view of the wholly different radiations and proteins used. However, it is realized that less clear cut results are to be expected with the smaller proteins because of their more rapid diffusion at a sedimenting boundary.

From the fact that the proportion of the particles affected by the x-rays decreased with increasing concentration and became negligibly small as the concentration approached 100 per cent, it is believed that many, or perhaps all, of the responsible "sensitizations" arose through the reaction of the hemocyanin with other molecules, presumably water, which had become "ionized." This view is also consistent with the observation, discussed below, that extraneous protein and high oxygen content each tends to inhibit the effect. Nor is it inconsistent with the observation that the total number of particles affected in a given volume of solution decreased as the concentration was decreased. A 15 per cent hemocyanin solution is, in terms of particle numbers, equivalent to 0.05 millimolar. Certain enzymes (8–11) show marked decreases in the total number of molecules affected by radiation as the concentrations are decreased below a few hundredths millimolar. The average lifetimes of some "ionized water molecules" are very short (2), especially in the presence of dissolved oxygen, and the proportion of them which was able to react with the hemocyanin probably decreased as these protein particles became more widely separated. Also, the average time that a hemocyanin particle will remain "activated," *i.e.* capable of attaching itself to another protein particle, may itself be very short.

The interpretation given above depends largely on the decrease in the proportional effect observed with increasing concentration of hemocyanin. It should be pointed out, however, that the observations which furnish the strongest support for this view were made on solutions centrifugally concentrated into gelatinous pellets (45 per cent concentration). Hemocyanin molecules in such a state probably suffer a great restriction in their usual Brownian movements and it is quite conceivable, if one assumes a limited lifetime for a sensi-

tization, that many hemocyanin particles failed to become associated before loss of reactivity.

An appreciable number of internal ionizations in the hemocyanin molecules must have occurred with the dosages used. Computations according to the method of Lea (27) indicate that even with 200,000 r about 40 per cent of the particles should have suffered internal ionizations (assuming, as does Lea, that ionizations occur in "clusters," averaging three per cluster). Most of these ionizations apparently produce no detectable effect. Apart from an unknown fraction of the principal effect which may, as just discussed, have been due to these ionizations, the only result which could very plausibly be attributed either partly or entirely to internal ionizations was the slight amount of dissociation or fragmentation. This could be detected with certainty only with the longer exposures. Incidentally, an inhibition afforded by these cleavage products could very well explain the slight lack of proportionality between amount of radiation and the amount of the principal effect.

Of interest in connection with internal ionizations is the work of Svedberg and Brohult (15). They observed that hemocyanin molecules of *Helix pomatia* were dissociated into halves by either ultraviolet light or α-rays, even when the temperature was lowered to that of liquid air. In the case of α-rays apparently nearly every molecule through which an α particle passed was split. That splitting was not observed as a principal effect in the present study may have been due to the different hemocyanin used, but was more probably due to the different kind of radiation used. Ultraviolet light is specifically absorbed by proteins and hence is not necessarily comparable with x-rays. The principal difference between α particles and x-rays of the type used in this study is the greater average density of ions along the path of an α particle as compared with that along the path of a primary electron. It is possible that this is the significant factor responsible for the occurrence of pronounced splitting in the experiments of Svedberg and Brohult as contrasted with the very small amount observed in our own.

The fact that Svedberg and Brohult apparently have not observed, along with the splitting by α particles of hemocyanin at room temperature, any heavier components comparable to those herewith reported is unexplained. However, this too can possibly be attributed to some difference between the two hemocyanins or the radiations used.

Although more than one hundred separate ultracentrifugations were performed in the present study, the data were not complete enough, nor were the experiments altogether of the right kind, to permit a precise picturization of the detailed processes which gave rise to all the phenomena noted. Certain experiments which might seem crucial were not performed because only when all the collected data had been carefully studied and correlated, and after the x-ray equipment was no longer available, were some of the pertinent fac-

tors appreciated. Nevertheless, it seems appropriate to discuss the most probable explanations which are consistent with the data.

The results clearly indicate the great importance of the oxygen tension during irradiation. As in the work of Fricke (3) with other materials, the inhibitory action of the oxygen presumably involves a direct reaction of the oxygen with ionized water molecules, but it is possible that oxygen may also compete with other hemocyanin particles in reacting with an "activated" hemocyanin molecule.

Following the work of Fricke (2) on simple compounds, Friedewald and Anderson (5) and Luria and Exner (7), among others, have shown that the presence of extraneous proteins reduces the observed effects of x-rays on virus and bacteriophage in aqueous media. Undoubtedly, the extraneous proteins used in the present study did compete with the hemocyanin by reacting with the activated water molecules. But, as in the case of oxygen, another possibility must be considered in explaining the observed inhibitory effect, which in the present instance is not necessarily an equivalent measure of the reduction in the damage sustained by the hemocyanin particles. The extraneous protein might well have competed with the *Limulus* protein by also combining with the activated hemocyanin particles. This, or a combination of some "activated" extraneous protein particles with unmodified hemocyanin, could very well explain the small apparent increase in the concentration of hemocyanin which was observed following irradiation in the presence of extraneous protein. According to this theory, one might expect, along with the apparent increase in concentration, a little additional spreading and a slight change in average sedimentation rate of the principal boundary (hemocyanin plus complex). If such effects were present, they were too small to be decisively differentiated in the present study, although the point was not thoroughly investigated through experiments properly designed for this specific purpose.

The reason for the greater inhibition of the principal effect accomplished in concentrated preparations of hemocyanin by the addition of undialyzed horse serum, as compared with egg albumin, is not clearly understood. It might be supposed that it was due to the presence of some low molecular weight substance, although the serum proteins themselves may have been responsible. Experiments with concentrated hemocyanin in the presence of dialyzed serum, which might have yielded some information on this point, unfortunately were not included in the present study.

The reversal of the proportional effect by the addition of albumin has an understandable significance when one considers that the production of the new ultracentrifugal components in any given preparation must depend not only on the number of hemocyanin particles which become "sensitized," but also on the average chance which each sensitized particle has of forming a stable combination with some other hemocyanin molecule. For those experi-

ments in which a fixed concentration of egg albumin was incorporated in the suspending medium, it is evident that the proportion of the initial hemocyanin which at some time during irradiation became "sensitized" could not have been greater in a concentrated preparation of hemocyanin than in a dilute one. Therefore, it may be concluded from the observed reversal of the proportional effect in the presence of albumin (2.8 per cent) that the fraction of the "sensitized" hemocyanin particles which was able to form stable combinations with other hemocyanin molecules was greater with high hemocyanin concentrations (15 per cent) than with low (0.8 per cent) ones. In other words, there must have existed some other competing "desensitization" process which assumed increasing relative importance as the rapidity with which the hemocyanin molecules could react with each other was decreased through a lowering of the concentration. Very probably some "desensitization" occurred through a reaction with the albumin molecules themselves. As already discussed, there was experimental evidence for some type of reaction between hemocyanin and albumin in irradiated mixtures.

In connection with the reversal phenomenon, attention should be called to the fact that since concentrated hemocyanin preparations were diluted with simple buffered saline for ultracentrifugation purposes, the concentration of the egg albumin did not in such cases remain at the fixed 2.8 per cent value used for the irradiation. Conceivably, though quite improbably, this could have had some bearing on the experimental results, and in any similar studies the protein should be incorporated in the diluent for optimum comparability.

From the observations on those irradiated preparations which were studied after heating, and again after subsequent storage, it is evident that under certain conditions the principal new component (86S) can be dissociated and at least partially reassociated. Although a breaking and reforming of some x-ray–created bonds may have been involved, it is sufficient and justifiable to assume that at least some individual hemocyanin molecules, even though in a state of combination with other hemocyanin molecules, were able to reversibly dissociate in a more or less normal manner. This view is supported by the fact that reassociation of the 86S component continued over a period of hours or days, whereas the combination of "sensitized" hemocyanin particles is known to take place very rapidly. Also, there was suggestive experimental evidence, immediately after heating, of some material of intermediate rate (between 60.5S and 86S) which presumably represented combinations that had lost less than a full molecular unit (60.5S) during the heating.

If the assumption is valid that some individual hemocyanin particles incorporated within combinations were able to dissociate and reassociate in a normal manner, then a possible explanation may be offered for the observed increase in the total concentration of the measurable components following the storage of unheated irradiated preparations. From statistical considera-

tions and the known facts regarding the dissociation-association process as related to hemocyanin, it can be argued that even at 4°C. very minute amounts of dissociation and association are continually taking place with an equilibrium so maintained that the concentration of the dissociation products never becomes great enough to be easily detected. Consider then as a limiting case a preparation which has been irradiated sufficiently to cause association or aggregation of all the primary hemocyanin particles. If this preparation is then stored for a long time, most of the primary particles will eventually have undergone at least one dissociation. However, many of the fragments dissociated from primary particles held together in aggregates will next combine to form individual unaggregated primary particles, the rate of recombination being very considerably greater in a concentrated preparation than in a dilute one. Also, if the larger aggregates become subdivided by the dissociation of some incorporated primary particle, some of the resulting smaller aggregates will associate with the elementary dissociation products of other primary particles. The net result will be a gradual decrease in the concentration of larger aggregates and an increase in the concentration of the simpler, more easily differentiated forms, particularly the unaggregated normal material. It can be shown theoretically, assuming that the dissociation and association constants of aggregated particles are not greatly different from those of normal hemocyanin, that this sort of transition is to be expected even when a considerable amount of the protein is originally in the unaggregated form, as was the case in the present experiments.

In conclusion, a new, easily measured, reproducible effect of x-rays on hemocyanin has been demonstrated. Several variables which influence the results have been studied in a preliminary manner. It is believed that through proper use of these variables the effect will be a useful means of studying quantitatively the reactions of at least this protein, including the reactions with ionized water, when it is irradiated with x-rays.

SUMMARY

1. When normal, monodisperse hemocyanin (60.5S) from *Limulus polyphemus* was irradiated in neutral buffer with x-rays, several new, more rapidly sedimenting ultracentrifugal components (86S, 107S, 122S) were produced, with a corresponding loss in the amount of the unaffected protein. The amount of the effect was roughly proportional to the amount of irradiation.

2. The new resolvable components apparently represented an association of the primary particles into aggregates of 2, 3, and 4 primary particles respectively.

3. The proportional amount of hemocyanin affected decreased almost to the vanishing point as the concentration of the protein was raised to high levels.

4. The absolute effect, *i.e.* the total number of particles affected in a given

volume, increased with the concentration of hemocyanin, at least for concentrations below 15 per cent.

5. The presence of 33 per cent horse serum during irradiation inhibited the effect on the hemocyanin almost completely, with hemocyanin concentrations of both 0.8 and 14 per cent.

6. The presence of 2.8 per cent egg albumin during irradiation lowered the effect by about 70 per cent in the case of dilute preparations (0.8 per cent hemocyanin), but by only about 25 per cent in the case of 14 per cent solutions.

7. A lowering of the solution's oxygen tension during irradiation enhanced the effect, almost doubling it in some cases.

8. The probable theoretical significance of these and other observations are discussed in the text.

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