Part IV. Enzymological Applications

THE IMPACT OF GEL ELECTROPHORESIS UPON OUR UNDERSTANDING OF THE ESTERASES*

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One approach that will lead to understanding the function of an enzyme is to determine precisely its location in the living system in which it operates. This approach has been used to study the nonspecific esterases since Gomori first introduced his technique for "lipase" in 1945. Since that time a number of histochemical methods have been introduced that have improved the sensitivity and precision with which this group of enzymes can be identified. The reports of workers using these methods are legion. Literature resulting from this work has provided us with good information about the topographical distribution of esterases but remarkably little concerning their function. One of the important factors contributing to our failure to learn more about the function of esterases is the fact that several esterase-active proteins exist in every species, and none of the histochemical methods demonstrate only one molecular species of esterase. This has resulted in our studying esterase activity in unknown mixtures in the cells of many living things. It is not surprising, therefore, that studies describing topographical distribution of esterases have contributed little to our understanding of their function.

The introduction of a method that combined electrophoretic and histochemical methods for the location and identification of esterase, by Pintér *et al.* (1954) using paper electrophoresis and by Hunter and Markert (1957) using starch gel electrophoresis, made it possible to understand more precisely the esterase composition of cells and tissues. This method, the zymogram method, when used to complement histochemical studies of esterases, should provide us with new information on which esterases are where and thus lead to more meaningful conclusions regarding their function. In this publication it is our purpose to report some of our recent work and to briefly review some of the work of others who are contributing to the study of esterases by these methods.

Materials and Methods

The materials and methods to be described relate to seven experiments which have in common only the use of the zymogram method to learn more about esterases. The technology involved has been described previously.§ In the work herein reported α-naphthyl butyrate was used as substrate and Blue RR

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§ By Hunter and Markert, 1957; Markert and Hunter, 1959; Hunter and Burstone, 1960; Hunter and Strachan, 1961; and Burstone, 1962. For further information see *References*.

salt as the diazonium salt in the histochemical reaction unless otherwise specifically noted.

1. *The effect of partial hepatectomy upon the esterase in mouse liver.* Female Swiss albino mice approximately seven weeks old were subjected to partial hepatectomy under sodium pentabarbital anesthesia. The portion that was removed, constituting 65 to 70 per cent of the liver, was weighed, diluted with 3 volumes of distilled water, homogenized and centrifuged at 15,000 × g for 10 minutes at room temperature. Following centrifugation the supernatant fluid was removed and analyzed, with an aliquot being frozen for future reference and comparison. At 17 hours and at daily intervals thereafter the mice were sacrificed and their livers removed. Care was taken to exclude the small bits of necrotic tissue near the ligature. The remaining liver was diluted with three volumes of water, homogenized and processed as the other samples previously described.

2. *Comparison of the esterases in mouse serum using starch-gel and acrylamide zymograms.* The starch-gel zymograms were prepared according to our usual methods, which were referred to earlier. The acrylamide gels were prepared according to the technique of Ornstein and Davis, with two important modifications. First, a larger amount of sample was applied to the columns. This consisted of mixing equal parts of serum and buffered sucrose solution. The sucrose solution was made up of 3 parts of 1 M. sucrose and 1 part of .06 M. tris (hydroxymethyl) amino methane-HC1 buffer, *p*H 6.7. The second modification was a reduction in the concentration of ammonium persulfate to a final concentration of 5.4×10^{-4} M. in both spacer and lower gels. Following the run the gels were incubated for 30 minutes at 37°C. in round, flat-bottomed tubes measuring 6 × 1.5 cm. and containing the histochemical reagents for esterase demonstration. Following incubation they were removed, washed with tap water, and returned to water-filled tubes for storage at room temperature. No changes have been observed in samples kept for several months.

3. *Esterases of human serum.* Serum or plasma samples were collected from more than 700 adults, stored for varying periods of time ranging from a few hours to 3 days at refrigerator temperature, and then introduced into starch-gel columns for electrophoretic separation according to our usual technique. Special care was taken to insure that the filter paper origins were all of the same size (5 × 13 mm.) and that 10 microliters were pipetted onto each. Half of each column was developed for esterase using α-naphthyl butyrate and Blue RR salt as previously described and the other half of the column stained for protein with Amido black as described by Smithies. Each of the esterase zymograms was photographed, and the protein bands seen in the pherograms were systemically recorded.

4. *Desmo and lyo esterases.* The liver was removed from a mouse and homogenized for three minutes in an all-glass homogenizer of the TenBrock type in three volumes of distilled water. Following homogenization the sample was centrifuged for 10 minutes at 10,000 × g, and the supernatant fluid was used to provide the material to saturate twelve 5 × 13 mm. pieces of filter paper. These pieces of paper were then inserted separately into 12 gel columns to serve as origins for 12 electrophoretic runs. After 4 hours of electrophoresis the filter papers that had served as origins were collected along with 2 mm. of the starch nearest the origin on the anodal side and placed in 0.5 ml. of distilled water overnight in the refrigerator. This material was then homogenized again and the solution absorbed onto new filter papers measuring 5 × 7 mm. to be inserted side by side with the original material on filter paper the same size. After four hours of electrophoresis the columns were sliced and developed for esterase.

5. *The effect of pilocarpine on esterases in the duodenum.* Young adult

Sprague Dawley rats were injected with pilocarpine in saline with a dosage of 1.6 mg. of pilocarpine per 100 gm. of rat. After 20 minutes the rats were sacrificed by being struck a quick blow on the head followed by decapitation. A segment of duodenum measuring approximately 1 cm. was removed, washed vigorously in running tap water, then slit open along one side and pinned out onto a large cork. The exposed mucosal surface was then scraped with a scalpal blade to obtain the epithelial tissue used in making the zymograms. The sample was homogenized in 4 volumes of distilled water and centrifuged at 10,000 × g for 10 minutes. The electrophoretic separation was carried out for 4 hours at room temperature and developed for esterases using α-naphthyl butyrate and naphthol AS acetate as substrates and Blue RR salt as the diazonium salt.

6. *The effect of pregnancy on esterases in rabbit serum.* Two groups of domestic rabbits, the first consisting of three rabbits and the second of two rabbits, were bred and the esterases in their serum studied according to the following schedule. Rabbits were bred on consecutive days, and the esterases and serum proteins analyzed 3 times weekly during the period of their pregnancy. Serum samples were saved in order to permit comparisons with previous days runs. The technique involved in running the material was the same as has been previously described in studying mouse serum (Hunter & Strachan, 1961), except that in the second group the sera were saved and compared in a vertical starch-gel system (Smithies, 1959), selecting the period where the esterase changes were most manifest. A third group of 3 animals were subjected to the aforementioned regimen but turned out not to be pregnant and did not show esterase changes.

7. *Esterase and plasma protein changes in the developing mouse.* Swiss mice from five late fetal stages, the newborn and postnatal mice from one through ten days and from two weeks to adult were examined. Over 100 individuals representing these 24 stages were sampled. Plasma and aqueous homogenates of heart, lung, brain, kidney, and liver were investigated.

In mice under one week of age the blood was collected in heparin-rinsed capillary tubes held next to the inferior vena cava, which was then ruptured by pinching with forceps. From mice over seven days the blood specimens were drawn from the inferior vena cava using a heparin-rinsed syringe and a 21 or 23 gauge needle. After centrifugation the plasma was removed with a Pasteur pipette and frozen until used. Organ samples were taken and frozen sealed in saran wrap. Where plasma or tissue samples were very small, specimens from litter mates were pooled.

Tissue samples from specimens under seven days were run undiluted, crushed evenly against the filter paper. Larger tissues were weighed and homogenized with an equal weight of water, homogenate then adsorbed onto the filter paper strip. Half of each column was stained for protein with Amido black; the other half was developed for esterase.

Results and Discussion

1. *The effect of partial hepatectomy upon the esterases in mouse liver.* Implicit in much of the work done on the compensating liver remaining in an animal following partial hepatectomy is the assumption that the unremoved liver undergoes, at least to some degree, a reversion to a more embryonic or uniquely proliferative condition such as we find in neoplasia. In fact, it was our thought that we might see a recapitulation of some of the liver esterase changes, associated with the developing liver, that are described elsewhere in this report. Ex-

amination of the results (FIGURE 1) reveal that, at least as far as the esterases are concerned, there is no regular or progressive change observable in the liver following partial hepatectomy. From this we conclude that once the cells of the liver have undergone the differentiation found in adult mice and assume the functioning of an adult mouse liver cell, they retain the same esterases, even after partial hepatectomy.

2. *Comparison of the esterases in mouse serum using starch-gel and acrylamide zymograms.* Mouse serum was used to prepare the esterase zymograms comparing starch gel with acrylamide gel (FIGURE 2). The numbered bands in starch gel correspond to the same bands identified earlier (Hunter & Strachan, 1961) in corresponding material. On the basis of their relative mobility, their intensity and sensitivity to eserine, we have concluded that the numbered bands in the acrylamide zymogram correspond to similarly numbered bands in starch gel.

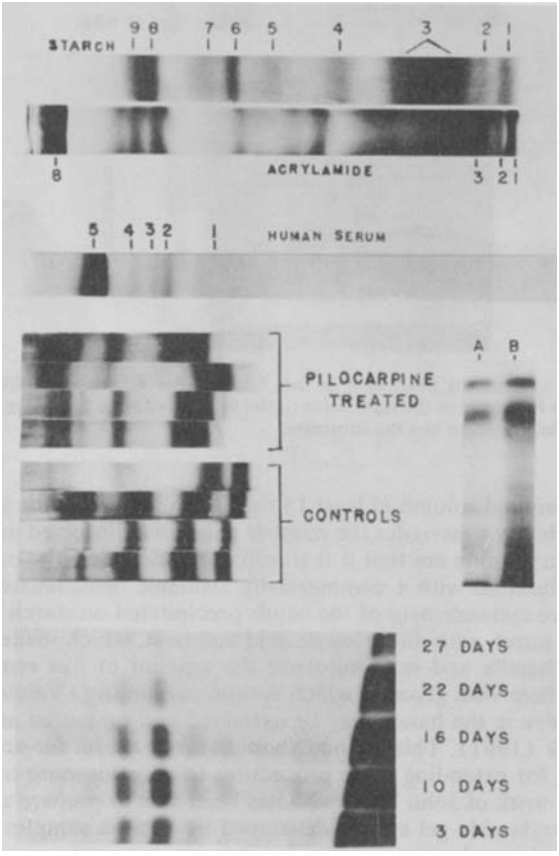

FIGURE 1. The two zymograms at the top compare the esterases seen in starch gel with those in acrylamide gel. The sample used was mouse serum, and the substrate was α-naphthyl butyrate. Below these is the human serum starch-gel zymogram illustrating 5 of the 6 commonly seen esterase-active proteins in human serum. The substrate used was α-naphthyl acetate. The two sets of zymograms at the left compare the esterases in rat duodenum in untreated and pilocarpine-injected animals. At the right, labeled *A* and *B*, are zymograms relating to the experiment on desmo and lyo esterases. At the bottom of the Figure is a zymogram illustrating the esterases in rabbit serum during pregnancy.

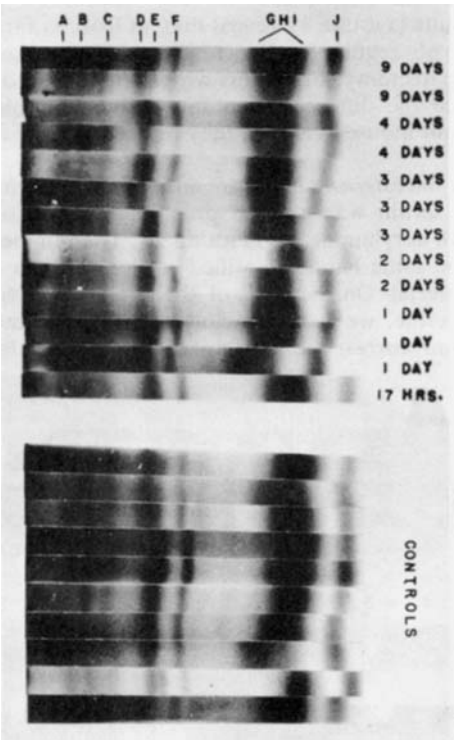

FIGURE 2. The zymograms illustrated in this Figure were all prepared from mouse liver. The hours and days indicated at the right refer to the interval of time following partial hepatectomy. Alpha-naphthyl butyrate was the substrate.

Examining the original column at least 15 bands can be seen in the acrylamide-gel column, which clearly supersedes the number that can be resolved in starch. Other advantages of acrylamide are that it is transparent and offers the opportunity for quantitative evaluations with a commercially available densitometer. In order to make quantitative measurement of the bands precipitated on starch it is necessary first to clear the starch with dilute acetic acid and heat, which makes the gels soft and difficult to handle and may influence the amount of dye remaining in the bands, or clear them with glycerol which is time consuming (Vahvaselkä, 1962). Alternately the dye in the bands may be extracted and measured as described by Wright and Keck (1961). This method should also be useful for acrylamide gels. The possibilities for extending these procedures to an even more micro scale are indicated by the work of John Pun* who has been able to prepare zymograms on a miniturized acrylamide-gel system developed by him on samples containing as little as 0.5 microliters of mouse serum.

3. *Esterases in human serum.* The purpose of this investigation was to observe any changes that may occur in the esterases in human serum as they may relate to disease. In only one instance were we able to observe a clear change in the esterase pattern in the serum examined and in this instance we were not able to see it again upon re-examination of the serum of the same patient or in other

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patients with similar symptomatology. Recent work indicates that human serum esterases can be more regularly and more clearly demonstrated if α-naphthyl acetate is used as substrate instead of α-naphthyl butryate as we used in this work. In addition it has been learned that increasing the amount of sample to twice that originally used has a beneficial effect (Lawler). Work is continuing along this line using these modifications.

4. *Desmo and lyo esterases.* One of the perennial questions that arises when the results of the application of electrophoretic gel methods are to be compared with the observed distribution of esterases in tissue sections is whether or not we are observing only the soluble (lyo) esterases as we work with electrophoresis and only the insoluble (desmo) esterases as we examine esterases in tissue sections as is conventionally done in tissue section histochemistry. This experiment was performed to determine whether the materials we often see remaining at the origin in zymograms is in fact composed of material different from that seen to have moved down the column. The desmo esterases can be compared to that remaining at the origin while the soluble esterases correspond to the esterases that have moved down the column. The material seen in the half column labeled "A" (FIGURE 2) represents the material that in the preliminary separation had remained at the origin. The material labeled "B" is from the original homogenate supernatant fluid. Comparison of the two reveals that the same esterases are present in both preparations. These results support our general thesis that the esterases seen in zymograms essentially reflect those present in the tissue or organ. Another observation that supports this conclusion is that of Petras (1963) in which he noted that in the case where a single prominent band was missing in the serum of a mouse this same band was also missing from organs normally containing the band. If the band had been absent from serum, where one might expect to find the more insoluble form that could be rendered soluble by extraction and homogenization, then one could more easily justify the contention that the esterases in zymograms do not reflect an accurate representation of the esterases in tissues and organs. We are under no illusions that we have proved that all tissue esterases are accurately reflected in zymograms; however, we do feel it is useful to assume that most of the esterases are demonstrated.

5. *The effect of pilocarpine on esterases in the duodenum.* The purpose of this experiment was to determine whether there was a characteristic change in the esterases of the duodenal mucosa following the injection of pilocarpine. At the time the animals were sacrificed it was obvious that the dosage was sufficient to cause considerable salivation and presumably secretion of other glands in the body. The principal difference observed in the zymograms was in the most rapidly moving band, which was prominently present in the control animals but was much reduced in concentration in the experimental animals. Other changes were also noted in the zymogram and in the cytology of the duodenal wall but were either not sufficiently reproducible or require further investigation before reporting. The differences reported in the fastest moving band could be illustrated with both α-naphthyl butyrate and naphthol AS acetate as substrates.

6. *The effect of pregnancy on esterases in rabbit serum.* The results of this experiment indicate that beginning on the 22nd day of pregnancy there is a progressive loss in certain of the esterases in rabbit serum that continues to and beyond parturition. The principal esterases involved are labeled "A" and "B" (FIGURE 2). A number of the other less prominent bands also seemed to be lost as the pregnancy developed, but because of the difficulty of observing them and the irregularity of their appearance it seems wise not to consider them further at present. It is an interesting observation that the serum esterase zymogram ob-

served in rabbits is considerably more variable than that of other animals we have examined extensively including mice, rats, and man. The zymogram to be obtained from an individual rabbit, however, was predictable and dependable from day to day. The individual differences observed in rabbit zymograms are not carried over to the pherogram patterns, which are much more similar to each other. Preliminary efforts to determine whether or not the observed changes in esterase were related to a change in serum protein concentration were conflicting and await further investigation. Examination of the protein stained starch-gel columns did not suggest a selective change in the concentration of the stainable proteins.

7. *Esterase and plasma protein changes in the developing mouse.* The purpose of this investigation was to determine whether the development of esterases and proteins was essentially a progressive phenomenon with the elements accumulating as differentiation proceeds, or whether instead there is a rise and fall in the kind and amount of esterases and other proteins that follow the progress of differentiation. While 5 tissues or organs, namely the liver, kidney, heart, lung, and serum, were investigated only two are illustrated (FIGURES 3 and 4) in this report, since it is felt that these are representative and demonstrate adequately the points we wish to make. In addition, the protein changes in serum have also been studied (FIGURE 5) and turned out to be of sufficient interest to warrant incorporation here.

In the liver (FIGURE 3) the background staining of the column indicating diffusely distributed esterase-active protein is present from the 0.4 gm. fetal stage on and in some specimens completely obscures a few of the bands. Dilution and

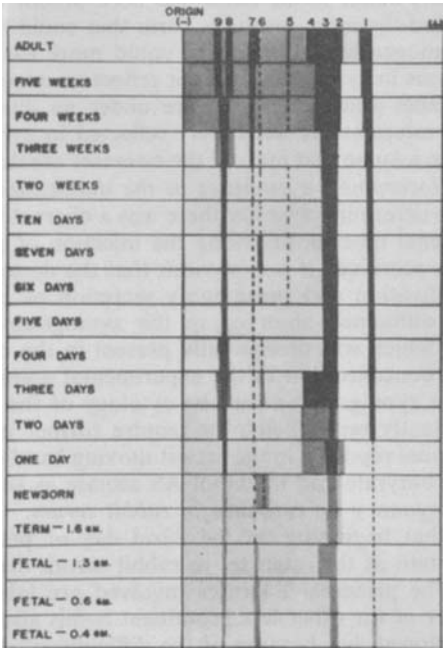

FIGURE 3. Diagram of the changes occurring in esterase zymograms during the development of the mouse liver.

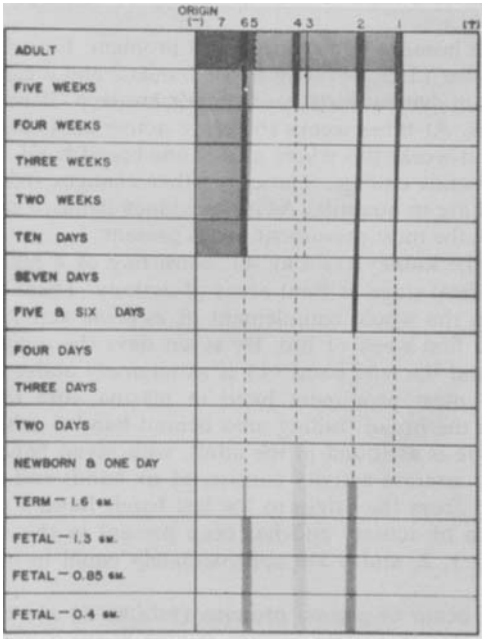

FIGURE 4. Diagram of the changes occurring in the esterase zymograms during the development of the mouse kidney.

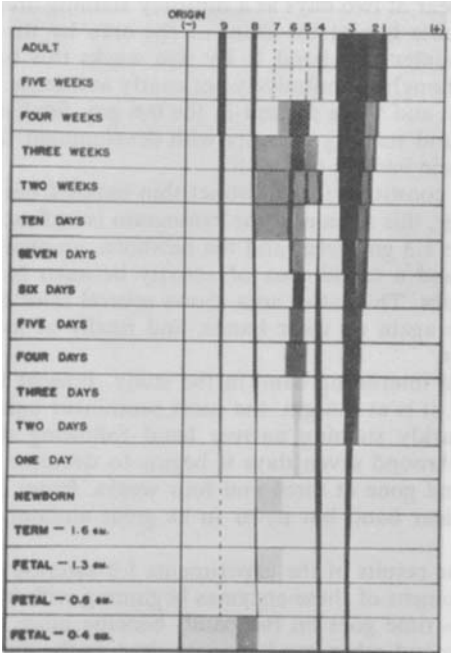

FIGURE 5. Diagram of the changes occurring in mouse plasma protein during development. Note particularly band 4.

centrifugation of the homogenate reduced this problem. Four bands are present in the 0.4 gm. fetal liver (1, 3, 6–7, and 8–9). Bands 2 and 4 can be distinguished from band 3 at 1.6 gm., and at birth seven bands are seen. Band 5 arises near the end of the first week. At three weeks there are active areas extending on either side of band 8, at four weeks this whole area is one broad band, and finally at five weeks two distinct bands emerge. The only other changes from the three-week picture to adulthood are in quantity. As in the kidney bands, 1 and 3 are approximately equal and are the most prominent bands present.

The esterases of the kidney (FIGURE 4), consisting of 4 main bands, are evident at the 0.4 gm. fetal stage as faint areas of activity. These four bands (1, 2, 4, and 6) constitute the whole complement of esterase activity throughout the fetal period and the first week of life. By seven days the active areas have become sharp bands and the lead band (1) is as intensely active as band 2, which corresponds to the most prominent band in plasma, and most of the other tissues. Band 3 and the broad trailing area behind band 6 arise at ten days. At two weeks the picture is as found in the adult, with seven bands present. In the adult mouse, kidney esterase activity consists of six bands and a broad streak of activity that extends from the origin to the last band. Band 5 appears faintly at the front of the area of activity and has been present in this position since the second week. Bands 1, 2, and 6 are approximately equal in esterase activity at this time.

The changes that occur in plasma proteins (FIGURE 5) are especially interesting. Since the adult plasma protein picture of nine bands is not defined until the fifth week and several interesting developments occur before then, the development of each protein band will be examined individually.

Bands 1 and 2 appear at two days as a diffusely staining area ahead of band 3. There is a more intense line at the front of the area by the fourth day which deepens and differentiates into band 1. By five weeks this band is as intensely staining as band 3 although quantitatively not nearly as intense.

Protein bands 3, 8, and 9 are present in the 0.6 gm. fetal stage and gradually increase in quantity and staining intensity with development. Band 3 is quantitatively the largest protein band in the adult.

Bands 5, 6, and 7 constitute three distinct thin bands from 5 weeks on. Prior to five weeks, however, this section of the zymogram is in flux: There is an active area at band 7 in the 1.3 gm. fetus and the newborn, an area at band 5–6 from one to seven days, and a broad area of activity between bands 4 and 8 from ten days to three weeks. This latter area shows several faint bands. At the four-week stage there are again no clear bands, and finally at five weeks a distinct adult picture emerges.

Band 4 is the most interesting band in the study. Present in the earliest fetal specimens examined, it is at 0.6 gm. the most prominent band. Throughout the first week it is a darkly staining narrow band following directly behind the albumin (band 3). Around seven days it begins to decrease in intensity, being faint at two weeks and gone at three and four weeks. From five weeks on it reappears as a faint clear band but never in as great amount as during the first week.

Examination of the results of the experiments for esterase reveal that there is a progressive development of these enzymes beginning with bands that are often weak and diffuse. As time goes on the bands become more discreet. They also become more intense and other bands appear. Just as the zymogram from each tissue is unique, so the order of esterase development is distinctly different for each organ. It is interesting that there is variation in the age at which the mature

complement of esterases is reached: kidney, 2 weeks; lung, 3 weeks; plasma, heart, and brain, 4 weeks; and liver, 5 weeks. Some of the esterase-active proteins are present in all the tissues examined. Others are common to a few. While no attempt was made to correlate each of the esterase-active bands in all the organs, it is apparent in this work as well as in previous work (Lemkey, 1962) that there are two esterases that are both strongly reactive and commonly present. These would correspond to bands 2 and 6 of kidney. Other bands seem to reflect a higher degree of specialization in certain tissues (band 9 in liver) and are likely to be associated with the special functioning of the tissue or organ involved.

General Discussion and Summary

The impact of gel electrophoresis upon our understanding of the esterases has been formidable in that it has provided us with new insights and understanding concerning the number and biochemical characteristics of the many esterase-active proteins found in biological material. The relationships between the esterases within a species and among species still remains largely to be determined. With regard to the function of esterases it is to be expected that there will be several. One promising possibility is suggested by the work of Allen and Hunter, which illustrated a dependent relationship between male sex hormone and the esterases in the mouse epididymis. Supporting this work is the observation by Shaw and Koen (1963) demonstrating the presence of an esterase in the mouse kidney, which also was dependent on male sex hormone. The change observed in the serum esterase of the pregnant rabbit reported here and in women by Friedman and Lapman (1961) may also relate to hormone changes associated with pregnancy, although this relationship remains to be demonstrated. A second area where the esterases are likely to be functioning is in relation to protein synthetic activity of the endoplasmic reticulum. The only evidence supporting this suggestion is the abundant presence of esterases found in this location. The seven experiments described and discussed here along with those included in the references may serve as illustrations of the kind of work that can be accomplished by the use of these methods.*

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