rate than that occurring in vivo. Then the central cells in the dentine papillae underwent necrosis, presumably as a result of nutritional insufficiencies. The cells of the enamel organ, over the next 10 days, progressively lost their characteristic appearance and reverted to a morphologically less differentiated type, eventually appearing as a thin layer of stratified epithelium covering the surface of the gelatin sponge.

In view of these extensive changes it was surprising that when this tissue was transplanted into syngenic hosts it had retained the potential to reaggregate into a tooth germ and to produce an almost perfectly formed mouse incisor tooth. It would seem unlikely that there were any epigenetic influences of an inductive nature derived from the host mice on the transplanted tissues, and therefore the potential to develop and differentiate must have been intrinsic.

It was not possible to determine the source of the mesenchyme in the induced teeth, but, as such tissue is known to be mesectodermal in origin (*9*) and specific to some degree as yet not entirely ascertained for tooth formation, it would seem that it must have originated in the transplanted tissue.

JAMES H. P. MAIN
Department of Dental Surgery,
University of Edinburgh,
Edinburgh 1, Scotland

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27 December 1965

Immunochemical Studies of Submicrosomal Membranes from Liver of Normal and Phenobarbital-Treated Rats

Abstract. *Rabbit antiserums were prepared against three submicrosomal fractions from liver of normal or phenobarbital-treated rats. The two membrane fractions originating from the rough- and smooth-surfaced endoplasmic reticulum were characterized by the same soluble antigens, with the exception of a highly basic component present only in extracts of rough membranes. The third fraction, whose subcellular origin is unknown, was different. It contained at least two typical marker antigens not present in the other fractions. Of eight tissue antigens common for the endoplasmic reticulum, five displayed nonspecific esterase activity. Some of these esterases were also found in other organs, but none was seen in rat serum. Phenobarbital treatment of the rats led to a rise in activity and characteristic changes in the esterase patterns of all these submicrosomal liver fractions.*

Extracts of isolated rat liver microsomes contain freshly synthesized antigens resembling the serum proteins and tissue antigens of unknown significance (*1*). Many of the latter are "cell fraction" as well as "liver" specific and originate mainly from the microsomal membranes of the parenchymal liver cells (*2*). The isolated microsomal fraction of rat liver is heterogenous with regard to subcellular origin and morphology. Isolated "plasma membranes" of rat liver cells (*3*), as well as those of Ehrlich ascites tumor cells (*4*), contain some antigens apparently distinguishing them from other elements of the microsomal fraction. We report an attempt to characterize immunochemically the "rough" and "smooth" membranes of the endoplasmic reticulum of rat liver cells (*5*), isolated from normal or phenobarbital-treated animals. Phenobarbital treatment leads to a specific rise in the activity of certain microsomal enzymes and a concomitant proliferation of the smooth-surfaced membranes of the endoplasmic reticulum of the parenchymal liver cells (*6, 7*).

Rat liver microsomes from starved Sprague-Dawley rats (both sexes) were subfractionated (*8*). Livers from groups

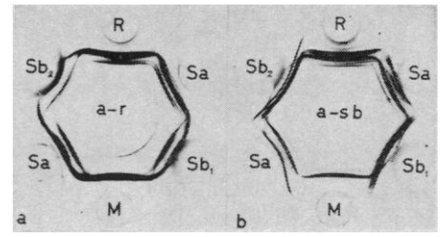

Fig. 1. Unstained precipitin reactions in agar. M, R, Sa, Sb_1, and Sb_2: detergent extracts of total rat liver microsomes, rough, Sa, and two independent preparations of Sb membranes, respectively. Protein concentration of all solutions, 6 to 8 mg/ml; a-r, a-sb: antiserums to rough and Sb membranes, respectively.

of five rats (total, 35 g) were homogenized in ice-cold 0.25*M* sucrose and centrifuged at 10,000*g* for 15 minutes to remove cell debris, nuclei, mitochondria, and lysosomes. Cesium chloride (15 m*M*) was added to the supernatant. It was layered over a 1.3*M* sucrose medium, containing 15m*M* CsCl; the tubes containing these solutions were centrifuged at 250,000*g* for 60 minutes (Christ ultracentrifuge, model Omega). The upper part of the supernatant, including the layer at the gradient boundary, was removed, and $MgCl_2$ was added to it to a final concentration of 10 m*M*. It was then again layered over sucrose (1.15*M*) containing 10 m*M* $MgCl_2$, and centrifuged at 250,000*g* for 30 minutes. The upper part of the supernatant, including the layer at the boundary, was again removed, diluted to 0.25*M* sucrose, and ultracentrifuged once more. In this way, three different fractions were prepared: (i) "rough" membranes, with attached ribosomes, which sediment in the presence of Cs^+ (first ultracentrifugal sediment, fraction R); (ii) "smooth" membranes which sediment in the presence of Mg^{2+} but not Cs^+ (second ultracentrifugal sediment, fraction Sa); and (iii) "smooth" membranes which were sedimented neither by Mg^{2+} nor by Cs^+ (third ultracentrifugal sediment, fraction Sb). Electron microscopical, chemical, and enzymatic studies have provided good evidence that the first and the second sediments originate from the rough- and smooth-surfaced parts of the endoplasmic reticulum, respectively. The origin of fraction Sb is uncertain (*8*). The three types of membrane fractions were injected into groups of four rabbits each. Each rabbit received three intramuscular injections, given at 3-week intervals, of 5 to 10 mg of pro-

tein, incorporated into complete Freund's adjuvant. Serums of four rabbits injected with the same preparation were pooled. For testing, membrane extracts were prepared by dissolving the isolated fractions with 1 percent sodium deoxycholate and 0.5 percent of the nonionic detergent lubrol W (*1*, *2*). Prior to use, all antiserums were exhaustively absorbed with lyophilized rat serum. Controls were set up with extracts of other subcellular fractions or other organs prepared as described previously.

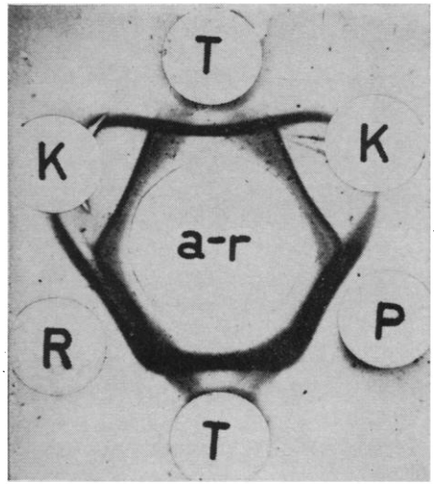

Fig. 2. Precipitin reactions in agar, stained for esterase activity. Only the esterase-active precipitates are visible here. T, K, and P: detergent extracts of rat testis, kidney, and parenchymal liver cells, respectively. Other symbols as in Fig. 1. Protein concentration of antigen, 10 mg/ml.

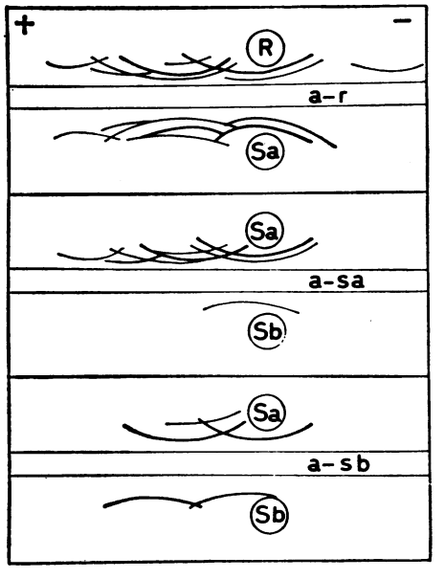

Fig. 3. Unstained precipitates appearing after immunoelectrophoresis in agarose (electrophoretic separation for 1 hour in barbiturate buffer, *p*H 8.2, 0.1*M*); a-sa: antiserum against Sa membranes. Other symbols as in Fig. 1. Protein concentration of antigen, 6 to 8 mg/ml.

When antiserums to the different membrane fractions were allowed to react with extracts of the various microsomal subfractions in agar diffusion plates, complex precipitation patterns appeared. In general, rough membranes and Sa membranes always rendered very similar pictures, while Sb membranes were different (Fig. 1). Most of the antigens shown were typical for the microsomal fraction (*1*, *2*).

To achieve a further characterization of the antigens, the precipitates were stained for nonspecific esterase activity with α-naphthyl proprionate (*9*) as substrate (Fig. 2). Extracts of rough microsomes contained at least three esterase-active antigens which were also present in a total homogenate of isolated parenchymal liver cells. Of these, at least one was present in an extract of rat kidney but not in that of testis, a second in testis but not in kidney, while the third was specific for parenchymal liver cells (*2*).

In subsequent experiments, the various extracts were subjected to immunoelectrophoresis in agarose. The extracts of rough and Sa membranes were identical with regard to the number and electrophoretic mobilities of the antigens (Fig. 3), with the exception of a cathodically migrating component present only in the rough membranes. The extracts of the Sb membranes were dissimilar and contained at least two antigens not present in the other fractions. At least five of the antigens present in the rough and Sa preparations displayed nonspecific esterase activity. Of these, one was present in kidney and liver, while another was found in testis and liver. Both were strongly stained and of low electrophoretic mobility. Only three esterases were present in low concentrations in the extracts of the Sb preparations, possibly because of contamination with other membranes. The two antigens typical for this fraction (Fig. 3) displayed no esterase activity. None of these esterases was found in rat serum.

For an immunological study of liver microsomes from phenobarbital-treated rats, groups of five female rats at a time were injected intraperitoneally for 4 days with 10 mg of phenobarbital per 100 g of body weight. After the last injection, they were starved overnight, and microsomal subfractions were prepared from their livers on the next day. This treatment led to a general increase of esterase activity in all three fractions, possibly because of uncomplete separation. However, the most conspicuous finding was the appearance

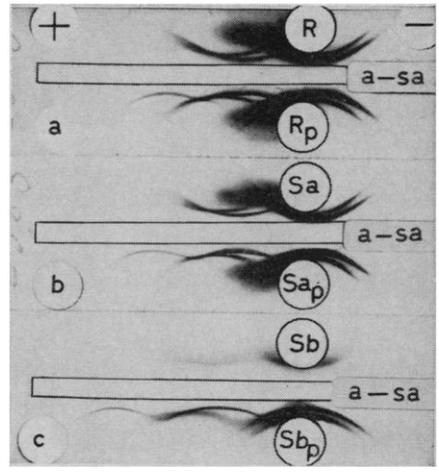

Fig. 4. Photograph of immunoelectrophoretic plates (*a*, *b*, and *c*) after staining of precipitates for esterase activity. Conditions for electrophoresis as for Fig. 3. R_p, Sa_p, Sb_p: detergent extracts of rough, Sa, and Sb membranes obtained from phenobarbital-treated rats. Other symbols as in Fig. 1. Protein concentration of antigen, 5 to 8 mg/ml.

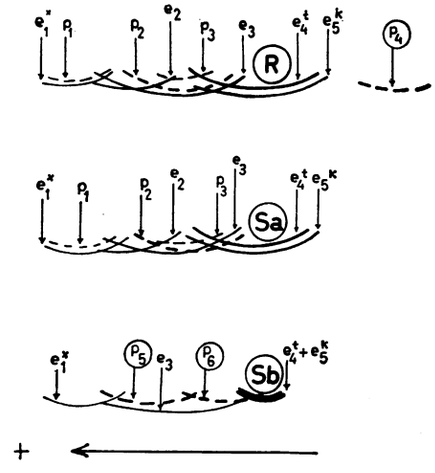

Fig. 5. Summary diagram of results of immunoelectrophoretic tests with the extracts of rough (R), Sa, and Sb membranes when tested with their homologous antiserums. Solid lines: esterase active antigens e_1 to e_5; e_1^x: phenobarbital-induced esterase; e_4^t: esterase present in liver and testis; e_5^k: esterase present in liver and kidney. Dashed lines: antigens p_1-p_6, displaying no esterase activity under the present conditions; p_4: antigen only found in extracts of rough membranes when reacted with antiserum to "rough"; p_5 and p_6: antigens only found in extracts of Sb membranes when reacted with the homologous antiserum. None of the antigens shown here was present in normal rat serum or red blood cells.

of a rapidly migrating antigen displaying esterase properties (Fig. 4). This esterase was hardly visible in normal membrane extracts and not at all in the microsomal supernatant ("cell sap") of total liver. Extracting it from parallel agar strips and injecting it into rabbits gave rise to the formation of antiserums rendering only one esterase-active precipitate, when tested with microsomal extracts. This precipitate was electrophoretically identical with the rapidly migrating enzyme seen in Fig. 4.

The great similarity between rough and Sa membranes (Fig. 5) agrees well with electron microscopical and biochemical findings (*8*). It could be assumed that the antigen which is specific for the rough membranes could be of ribosomal origin. So far RNA has not been identified in this component.

It is not surprising to find a variety of microsomal esterases, since liver microsomes contain many hydrolytic enzymes (*10*). Although some of our esterases may represent multiple aggregates of the same protein or the same enzyme protein adsorbed to different carrier proteins, others such as e_4^t, e_5^k, and e_1^x most probably constitute different molecular entities. From the evidence at hand it cannot yet be stated to what extent they represent isoenzymes. Phenobarbital-stimulated induction of esterases hydrolyzing various drugs has been observed (*7*). However, the nature of our phenobarbital-induced esterase e_1^x is as yet unknown. The typical electrophoretic mobility of this enzyme makes it particularly suitable for separation and further biochemical studies.

An important finding of our study was the deviating composition of the extracts of the Sb membranes. Our findings are in agreement with those of Dallner who found that the enzymatic composition of this fraction was qualitatively and quantitatively different from that of the other submicrosomal fractions. The question of the subcellular localization of this fraction is unsettled, and it is not even known whether or not it is of parenchymal origin. However, the presence of two typical marker antigens (p_5 and p_6, Fig. 5) should greatly facilitate its further characterization.

ULF LUNDKVIST
PETER PERLMANN
Wenner-Gren Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden

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Steroids and Serum Complement in Mice: Influence of Hydrocortisone, Diethylstilbestrol, and Testosterone

Abstract. *Hydrocortisone depresses hemolytic complement in male and female mice. Testosterone causes increase of serum complement in female mice, and diethylstilbestrol causes decrease of serum complement in male mice, in each instance to activities approximating those found normally in the opposite sex. Male and female sex hormones have no effect, in the doses used, on the serum complement of male and female mice respectively.*

Complement (C′), a group of naturally occurring serum proteins, is believed to enhance immunological reactions. Among the endocrine organs which have been studied for an effect on the complement system are the hypophysis (*1*), the gonads (*2*), the thyroid gland (*1, 3*), and the adrenal gland (*1, 4*). No coherent picture emerges from these studies, and for good reason. Difficulties are encountered in measuring effects on the complement system, since it consists of at least nine distinct serum proteins, five of which have but recently been recognized. Changes in the concentration of one or more of these components may or may not be detected in any particular assay of complement.

There is a well-documented difference in amounts of C′ in male and female mice (*5, 6*). Since this difference suggests the possibility of a hormonal control of a C′ component determinative of the C′ titer, as measured by hemolytic assay, this species offers favorable material to study. We report on the effects of relatively large doses of an androgen, a synthetic estrogen, and an adrenocortical steroid on hemolytic complement action in mice. The accompanying report of Weintraub *et al.* (*7*) supplements our study and goes beyond it in attributing some of the observed effects to one or more of the late-acting C′ components.

Serum C′ levels are expressed as units of C′ per milliliter which yielded 50 percent lysis of a standard number of red blood cells sensitized with antibody (*5*). The dosage, vehicle, and route of administration of hormone are indicated for each experiment. The mice used were the inbred C57Bl/10-h-2d (new line) raised in this department

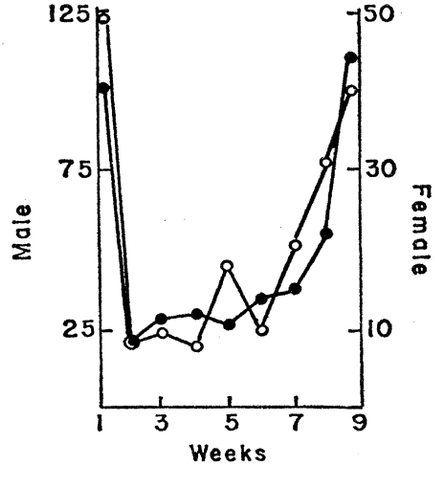

Fig. 1. The depression of serum C′ in male and female mice as a result of a single injection (sc) of 10 mg of hydrocortisone. *Abscissa*: Time in weeks after treatment. *Ordinate*: Mean titers ($C'H_{50}$ units per milliliter) in serum of male (left) and female (right) mice. ○——○, Average of six male mice determined each point; ●——●, average of three female mice determined each point.