

Ontogenesis of serum esterases in *Mus musculus*

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INTRODUCTION

Electrophoretic techniques by which mixtures of isoenzymes, and homologous proteins generally, may be resolved have become valuable tools in developmental biology. They make possible the study of development at the molecular level, in the sense that the differentiation of protein patterns underlies ontogenetic changes and tissue differences. The greater successes of this approach have been achieved, of course, in the fields of haemoglobin variation and of tissue-specific isoenzymes of lactic dehydrogenase (see Ingram, 1961; Markert & Ursprung, 1962).

The work reported here concerns proteins characterized by their ability to catalyse the hydrolysis of esters. Their substrates range from carboxylic to aromatic esters, and there is a corresponding variety of esterases. It is usual, however, to find that each is effective, though to a different extent, on more than one form of esters. The overlap makes it legitimate, and in fact necessary, to study esterases as a group, although phosphatases are usually dealt with separately.

The esterases of the mouse serum have been the subject of several studies in the past, but the need remained for an investigation combining improved electrophoretic resolution with a comparison of ontogenetic stages.

MATERIALS AND METHODS

Blood samples

To avoid the possible complication of genetic variation, only blood samples from mice (*Mus musculus*) of one laboratory strain, C3H, were used. The samples were obtained by decapitation, or from the tip of the tail, under ether anaesthesia. The serum was separated by centrifuging the heparinized blood. In all, over 500 samples were examined.

Starch-gel electrophoresis

Starch gels were prepared from hydrolysed starch (Connaught) and the electrophoretic runs were continued for 2.5 h at 15-20 mV/cm length, and 2-4 mA/cm width of the starch plate.

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The degree of resolution achieved in the zymogram depends on the duration and conditions of electrophoresis, and on the buffer system. Following various tests, we have found the following two buffer systems to be satisfactory with our material:

System 1	System 2
Gel buffer pH 7·6	Gel buffer 8·6
10·5 g/l citric acid	0·5 g/l citric acid
92·0 g/l Tris	2·7 g/l Tris
	(plus 10 % of vessel buffer)
Vessel buffer pH 8·6	Vessel buffer pH 8·6
18·60 g/l boric acid	11·8 g/l boric acid
4·0 g/l NaOH	1·2 g/l NaOH

For the composition of 'System 1' we are indebted to Dr J. W. B. King (Animal Breeding and Genetics Research Organization, Edinburgh).

Enzymic tests

Gel slices were stained for esterases by a widely used method adapted from the histochemical technique of Nachlas & Seligman (1949). The gel is incubated in a buffered solution with the substrate, the latter in the form of a naphthyl or naphthol ester. The enzymes, if present, release naphthol. This then couples with the dye, Fast Garnet, also added to the medium, to produce a purple precipitate.

Substrates used in this study include:

Carboxyl esters

Non-specific	1-naphthyl acetate
Phosphate	1-naphthyl phosphate
Choline	6-bromo-2-naphthoxy choline iodide
Short-chain fatty acid	1-naphthyl butyrate
Long-chain fatty acid	2-naphthyl laurate
	2-naphthyl stearate

Aromatic ester

Naphthol-AS-acetate

The non-specific substrate reveals numerous zones of activity in the stained gel (zymogram) but many of these fail to react with some or all other substrates.

Inhibitors added to the incubating medium may also affect various esterase zones selectively. We have used the following inhibitors:

Eserine compounds	Neostigmine
	Physostigmine sulphate
Organophosphates	Di-isopropyl fluorophosphate (DFP)
	Diethyl- <i>p</i> -nitrophenyl phosphate (E 600)

Organophosphates (<i>continued</i>)	Diethyl-3-chloro-4-methyl coumarin-7-yl phosphate (Coroxon) Di-(2-chloroethyl)-3-chloro-4-methyl coumarin-7-yl phosphate (Haloxon)
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The results of tests with a range of substrates and inhibitors help distinguish functional esterase groups: phosphatases, cholinesterases, aliesterases (including lipases), aromatic esterases (aryl-esterases).

Some gel slices were stained in nigrosine (4 % in methanol-acetic acid-water, 40:10:50). This reveals protein zones irrespective of enzymic properties, but most esterase fractions, due to their low amounts, fail to produce a nigrosine-stained zone.

The stained gels were photographed by transmitted light in the case of zymograms, and by reflected light in the case of general protein patterns.

Comparability of zymograms

Esterase zones are of unequal intensity. To detect the fainter fractions, incubation has to be prolonged, and the serum sample increased (a thicker filter paper is used for this purpose as sample holder). As a result, the stronger fractions are 'over-stained' and may fuse with, or cover up, other neighbouring bands.

Furthermore, electrophoretic conditions may have to be varied in order to obtain separation of certain fractions that otherwise appear as a single band.

Examples of the application of this flexibility of technique are given and explained in Plate 1 A and B. The conditions varied were length of run and dilution of the buffer. It follows that zymograms run under different conditions will show *apparent* differences.

RESULTS

Mouse serum gives a basic pattern of 19 esterase zones. As explained, it is very difficult to show all these clearly on one zymogram and one photograph; Plate 1 A is a good approximation. These fractions are described in detail elsewhere (Arnason & Pantelouris, 1966). The basic pattern does not apply to the foetus nor to the very young animal; it is also modified by pregnancy in the female, and shows certain other differences in the two sexes.

Foetus

The major 'adult' esterases are detectable in the serum of the 16- to 18-day foetus. Fractions 16-19 are in fact stronger in the foetal serum than in the adult, whilst for most fractions the reverse is the case (see Table 1).

In addition, however, the foetal serum comprises six esterase fractions that are absent in the adult (though they persist for a short time after birth). These, marked F 1 to F 6 are shown in Plate 1 B. The last fraction, F 6, is very slow and

it often proves difficult to get it to move away from the origin (i.e. the point of insertion of the samples) so that it is often missed.

F5 is lost within a day or two from birth, F1, 2, 3, 6 in the second week, but F4 may be present in the zymogram up to 2-3 weeks (see Plate 1B).

A long electrophoretic run is necessary to show F1 and F2 as separate fractions. In shorter runs they form a single band which, moreover, coincides with the nigrosine-staining foetal protein to which attention was drawn by Pantelouris & Hale (1962) (Plate 2A). This foetal protein decreases as the albumin increases. F3 and F4 have corresponding nigrosine bands, as shown in the same illustration, but F5 and F6 have not.

All six foetal esterase fractions react with naphthyl acetate and naphthyl butyrate. In addition, F3 and F4 hydrolyse 6-bromo-2-naphthoxy choline iodide and F4 hydrolyses naphthol acetate as well. Eserine compounds and organophosphates cause partial inhibition of F3 and F4.

Alkaline phosphatase is readily detectable in the foetal serum, in the form of a single or double slow band, but is not found in the adult. It can be shown that it persists for up to a month from birth (Plate 2B).

Pregnancy

The following, illustrated in Plate 3A, are differences consistently found between pregnant and non-pregnant females:

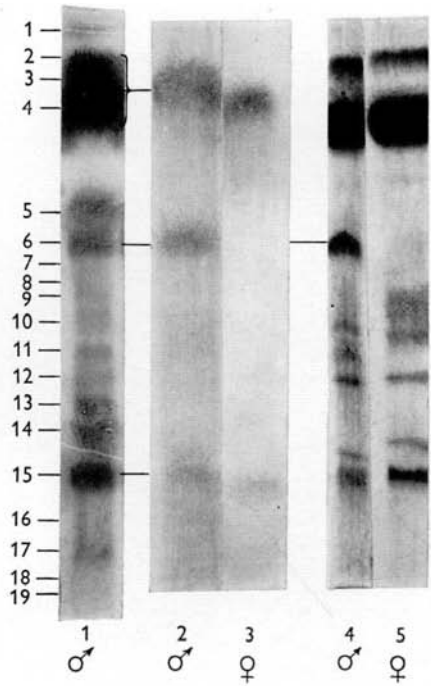
Fractions 5, 10 and 11 *increase* in pregnancy, reaching a peak in the second

PLATE 1A

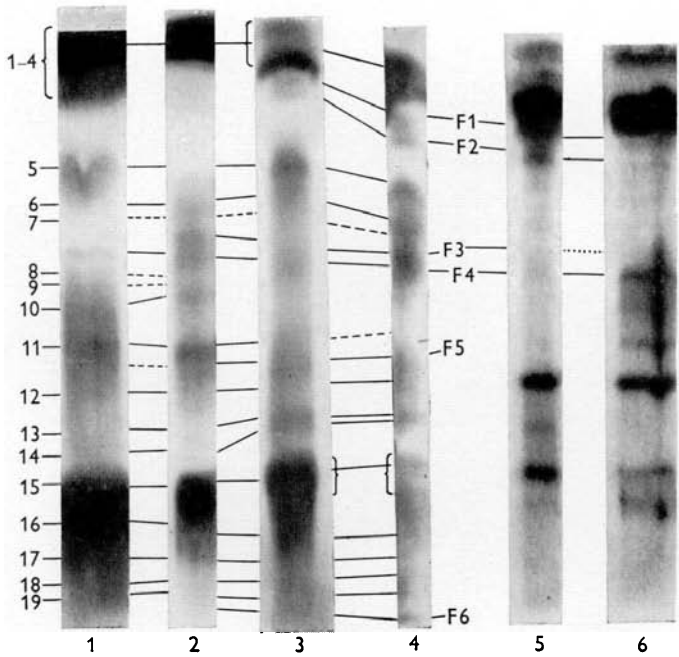
1. Zymogram showing the basic pattern of 19 esterase zones in the adult mouse serum. Buffer system 1, pH 7.6. Substrate: 1-naphthyl acetate.
2. Male adult mouse serum. An acid phosphatase zone overlapping with esterase 6. Buffer system 1. Incubation in veronal acetate buffer pH 5.2. Substrate: 1-naphthyl phosphate.
3. Female adult mouse serum. Note that the acid phosphatase of 2 is absent.
4. Male adult mouse serum. Total-protein staining with nigrosine. Note the strong protein band overlapping with esterase 6. Buffer system 1, pH 7.6.
5. Female adult mouse serum run concurrently with 4. Note the absence of the strong protein band found at the esterase 6 position in the male.

PLATE 1B

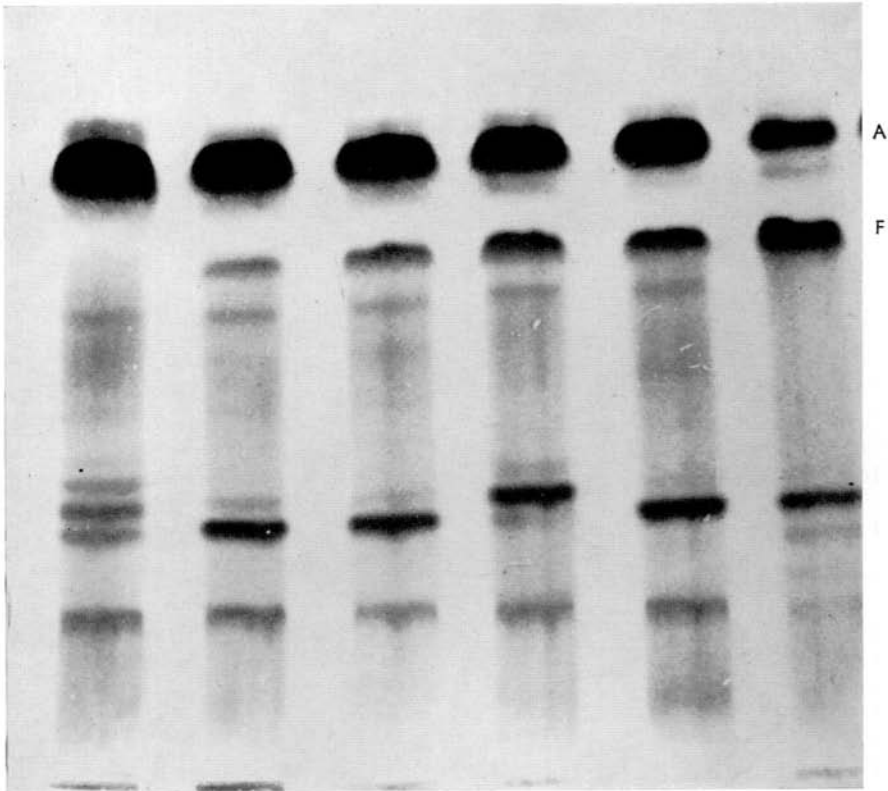
- 1 and 2. Esterase zymograms of young sera. Buffer system 1. Substrate: 1-naphthyl acetate. Starch 10 g/100 ml of buffer. The buffer was diluted 1:1. At this concentration of the buffer, esterase fractions 1-4 and F1-2 fuse into one broad zone. Ages of animals: 1, 14 days after birth; 2, newborn. Note absence of F3 in the former.
- 3 and 4. Buffer of normal concentration. Starch 13 g/100 ml of buffer. This system improves resolution at the two ends of the zymogram, but F3 and F4 fuse. Note the resolution of F1 and F2. Ages: 3, 18-day foetus; 4, 16-day foetus.
- 5 and 6. Strips from the same starch plate as 3 and 4 stained for total protein with nigrosine. Note the correspondence of F1 and F2 to two nigrosine fractions. Under the conditions of Plate 2A these two nigrosine fractions appear as a single foetal protein. F3 and F4 also have corresponding fractions. F4 persists in the 14-day old, whilst F3 is missing (see also Table 1). Ages: 5, 16-day foetus; 6, 14-day-old young.



A



B



A

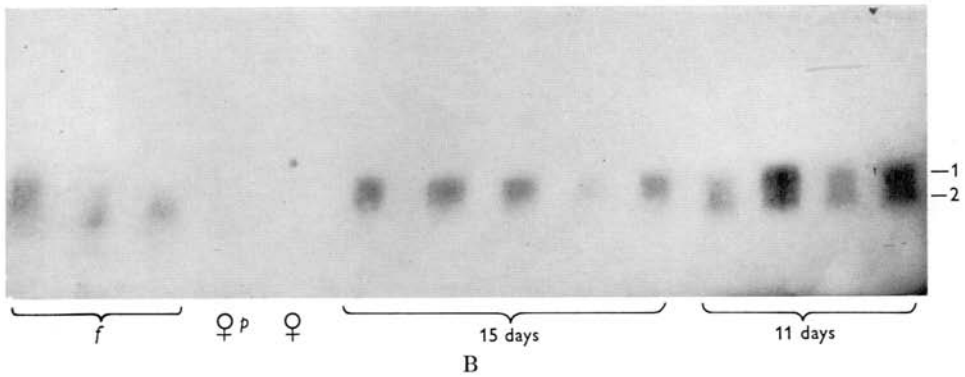


PLATE 2A

Nigrosine-stained serum electropherograms of (from left to right): 18-day foetus, 1-day, 4-day, 4-day again, 8-day and 19-day young. Note the decrease of the foetal protein F and the increase of albumin A. Buffer system 2, pH 8.6.

PLATE 2B

Alkaline phosphatase in mouse serum. Buffer system 2, pH 8.6. Substrate: 1-naphthyl phosphate. Note the presence of 1 or 2 bands of phosphatase activity in 16-day foetal sera *f*, and in 11-day-old and 15-day-old animals. No phosphatase can be detected in the sera of the adult pregnant (♀*p*) and non-gravid (♀) female.

Table 1. *Esterase fractions in mouse serum*

Fraction	Age (days)						
	Foetus	1	2-7	8-14	15-21	22-28	29-50
1	±	±	±	+	+	++	++
2	±	±	+	+	+	+	++
3	±	±	+	+	+	+	++
4	+	+	++	+++	+++	+++	+++
F1, 2	++	+	+	±	.	.	.
5	±	±	+	+	+	+	+
6	±	±	±	±	+	+	++
7	.	±	±	±	±	±	+
F3	+	+	±	±	(±)	.	.
F4	±	±	+	+	+	±	.
8	.	.	±	±	±	+	+
9	±	±	±	±	±	+	+
10	±	±	±	±	±	+	+
P1	.	(±)	(±)	.	.	.	(++)
11	.	±	±	+	+	++	++
F5	+	±
12	±	±	±	±	+	+	+
13	±	±	±	+	+	+	++
14	±	±	±	+	+	+	+
15	±	+	++	++	++	++	++
16	+	±	±	±	±	±	±
17	+	+	+	+	+	+	+
18	+	±	±	±	±	±	±
19	+	±	±	±	±	±	±
F6	+	±	±

week. Whilst 11 returns to normal after parturition, 5 and 10 do not do so until the twentieth day of lactation.

Two esterases, 8 and 9, *decrease* in amount. The former disappears altogether by the thirteenth day of pregnancy, and does not return to its normal level until 11 days after parturition.

The strong esterase fraction marked P1 is specific to pregnancy. It appears on the fourth day and reaches a peak in the tenth to fourteenth days of pregnancy, stays at that level until parturition and may persist for another 10-14 days. This 'pregnancy esterase' hydrolyses naphthyl acetate, naphthyl butyrate, 6-bromonaphthyl choline and naphthol-AS-acetate, but not naphthyl phosphate, stearate or laurate. All organophosphates tested (except TOCP) inhibit it, whilst eserine compounds do so only partly.

Male

The esterase 'fraction' 6 is found to be consistently stronger in adult males than females, and a further effort was made to test whether it is indeed a single fraction. By a longer electrophoretic run (12 cm), and a reduction of voltage (to avoid overheating), it was shown to comprise three separate fractions, denoted as 6A, 6B and 6C. The first two of these are common to both sexes, but the third is only found in mature males, and is absent from young males as well as all females (Plate 3B).

The three are clearly distinct enzymes, in that the first is inhibited by eserine compounds and esters of choline are its preferred substrate; the second is inhibited by organophosphates and hydrolyses naphthol acetate; the third resists organophosphates.

When naphthyl phosphate is used as the substrate (at pH 5.2) a prominent acid phosphatase band is revealed with the same mobility as 6C. It also is seen only in males (Plate 1A) and may indeed be identical with 6C.

DISCUSSION

The work reported here, together with other studies of this series (Arnason & Pantelouris, 1966; Pantelouris & Arnason, 1966) provide a basis for investigations on the frequency, physiological role and inheritance of esterase variation.

The ontogenetic changes and differences demonstrated include: the presence in the foetus of 5-6 fractions that disappear in the adult; the existence of a 'pregnancy esterase': and the presence in the male of another esterase and an acid phosphatase exclusive to that sex (these two overlap electrophoretically, and may be one molecular species).

In addition, there are quantitative changes: esterases 16-19, though not

PLATE 3A

Serum esterase zymograms of non-gravid female (*np*), 9-, 12- and 17-day pregnant females, and at 2, 9 and 12 days of lactation. Note the decrease of the 'pregnancy esterase', P1. Buffer system 1, pH 7.6. Substrate: 1-naphthyl acetate.

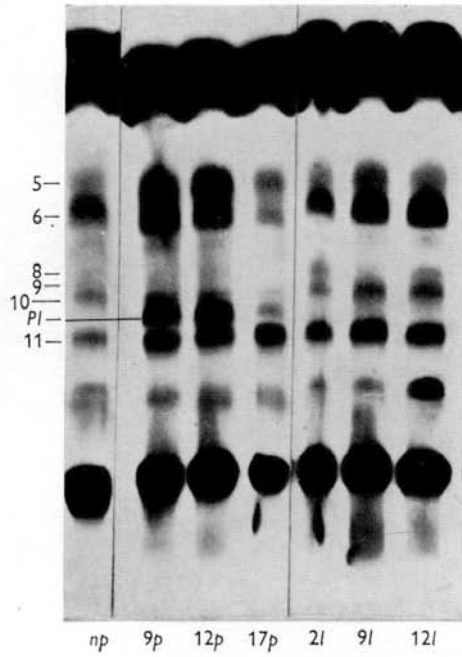
PLATE 3B

Sex differences in the serum esterase zymogram. Buffer system 1.

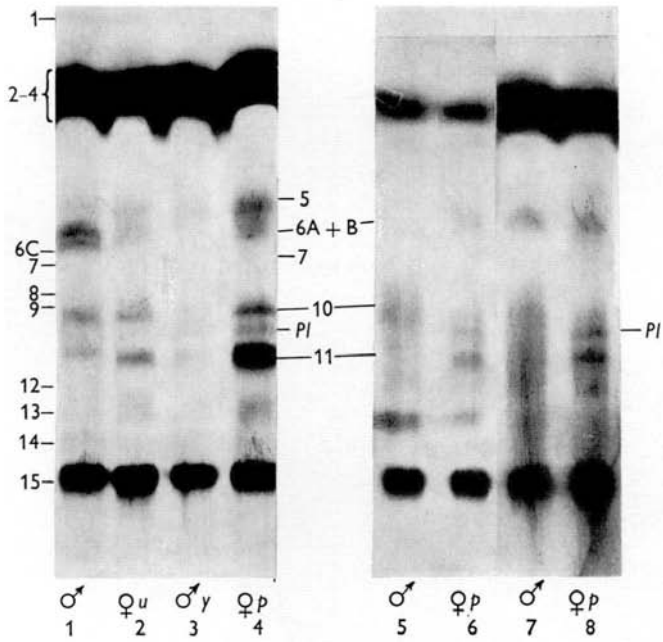
1 to 4. Adult male, non-gravid female, immature male and pregnant female. Note in 1 the separation of 6C from 6A + 6B; the location of P1 and its presence in the pregnant but not the other three animals; the large increases of 5, 10 and 11 in pregnancy. Substrate: 1-naphthyl acetate.

5 and 6. Adult male and pregnant female. Substrate: 6-bromo-naphthoxy choline iodide. Note the difference particularly in esterase 11, and also the presence of cholinesterase-type of activity in the pregnancy fraction.

7 and 8. Adult male and pregnant female as in 5 and 6. Substrate: 1-naphthol-AS-acetate. Note that P1 hydrolyses all three substrates represented in this illustration.



A



B

exclusively foetal, are stronger before than after birth. In pregnancy, fractions 5, 10 and 11 increase whilst 8 and 9 decrease in amount. The 'male esterase' (fraction 6C) is only found in the sexually mature male.

The tests carried out with a range of substrates and inhibitors help to distinguish between functionally different esterases. It was thus shown that fractions 6A, 6B and 6C are not isoenzymes. Their substrate range is different. Also the first is sensitive to eserine, the second to organophosphates and the third is unaffected by both groups of inhibitors.

Similarly, foetal esterase F3 exhibits activity of the cholinesterase type whilst F4 behaves more like an aromatic esterase (aryl or A-esterase). Unfortunately, the low concentration of these foetal fractions makes thorough testing with alternative substrates and inhibitors difficult. It does, however, appear that these fractions are rather unspecialized in substrate preferences.

The 'pregnancy esterase' is partly eserine-sensitive and exhibits enzymic activity of the cholinesterase type; it also acts on an aromatic ester, naphthol acetate, but not on fatty acid esters. It is interesting that it is totally inhibited by at least some organophosphates. It is never found in males, and was seen only in two out of 300 non-gravid females present, whilst it is very obvious in all pregnant females. The two apparent exceptions are readily explained by assuming interrupted pregnancies. It is situated between adult fractions 10 and 11.

Quantitative changes in mouse serum esterases associated with pregnancy have also been reported by Cons & Glass (1963) who did not, however, detect the specific pregnancy esterase. These authors used Smithies's borate buffer pH 8.6, compared to our discontinuous 'system 1' of gel pH 7.6 and vessel pH 8.7. At gel pH 8.6 this fraction is replaced by a smear in about the same region.

From the developmental point of view, the instances of 'switching over' from certain foetal to certain adult proteins are of particular interest. A theory concerning the mechanism of such changes suggests that the product of one operon may serve as a repressor of the activity of another. Such interactions between operons, via their products, are now widely discussed (Waddington, 1962—'cascade repression'; Pontecorvo, 1963; Stent, 1964; Gruber & Campagne, 1965; Manwell, 1966). The evidence has come mainly from micro-organisms (see Bacon & Vogel, 1963), but the applicability of the model to hemoglobino-pathies is also arguable (Zuckerkindl, 1963).

It is known that in at least some cases, enzyme differentiation is related to the hormonal make-up of the developing animal. There are several examples of hormones affecting the synthesis of particular serum proteins and enzymes. Augustinsson (1961) found that the level of serum aryl-esterases is lower in the boar than in the piglet, but can be raised by castration, and again lowered by testosterone injections. The level of ceruloplasmin in chick serum (Starcher & Hill, 1965) and of serum proteins in the hamster (Ditzel & Hove, 1965) can be changed experimentally by hormone administration.

In view of the above, it may be worth considering whether information gained in this study fits in with the requirements of theory; and whether any of the ontogenetic changes described provide suitable systems on which to test the idea of regulation of synthesis of one protein via changes in the level of hormones and in the rate of synthesis of another protein.

Regulation by hormonal factors of the pregnancy esterase, the specific male esterase and acid phosphatase, and of the quantitative sex differences described, is quite likely; and this speculation could be put to the experimental test.

The foetal protein-albumin system illustrated in Plate 2A is a case of one protein decreasing gradually as another increases. It would be rash to propose from this that albumin acts as a repressor of fetuin, but the possibility is worth considering (this point is discussed more thoroughly by Zuckerkandl, 1964). Should a regulatory interaction between genes responsible for the two proteins exist, it would be expected that analbuminemic mice (if discovered) should possess an excess of fetuin.

Foetal esterases were also shown to decrease after birth gradually. F4, for example, remains detectable for up to 4 weeks, whilst its neighbouring 'adult' fraction 8 appears in the first week of life and rises to its maximum over the same period. It is proposed to measure these quantitative changes accurately in normal development. It might also prove possible to accelerate or delay maturation of the young animal by hormonal treatment, and to follow any alterations in the amounts of various esterase fractions following such treatment. Experiments along these lines may provide useful evidence bearing on the question whether the inverse changes in the amount of certain 'foetal' and 'adult' esterase fractions are causally independent or interrelated.

SUMMARY

1. The 'typical' serum esterase zymogram, obtained by starch-gel electrophoresis, of adult mouse serum comprises 19 fractions, but undergoes changes related to developmental stage.

2. The main adult fractions are already present in the 18-day foetus. Whilst most are weaker than in the adult, the reverse is true for fractions 16-19.

3. There are also six 'foetal esterases' which gradually decrease and disappear after birth, at different rates. The foetus and the young animal, up to 2 weeks, have also alkaline phosphatase (1 or 2 fractions), whilst no such enzyme was detectable in the adult's serum.

4. Pregnancy is associated with increases in three and decreases in two adult fractions and, more characteristically, with the appearance of a strong specific pregnancy esterase.

5. An esterase zone (6) which is always stronger in the male could be subdivided into three fractions in males and two in females. The exclusively male fraction coincides with a band of acid phosphatase activity.

RÉSUMÉ

Ontogenèse des estérases chez la souris (Mus musculus)

1. Le zymogramme typique des estérases du sérum de souris adultes comprend 19 fractions, mais subit des modifications en rapport avec la stade de développement.
2. La plupart des fractions adultes sont présentes dans le fœtus de 18 jours. Alors que la majorité d'entre elles sont beaucoup plus faibles que chez l'adulte, l'inverse est vrai pour les fractions 16 à 19.
3. Il y a aussi 6 'estérases foetales' qui disparaissent graduellement après la naissance.
4. La gestation est associée à l'accroissement de trois fractions et à la diminution de deux autres, et, de manière plus caractéristique, à l'apparition d'une estérase 'forte', spécifique de la gestation.
5. La fraction 6 est plus forte chez les mâles et peut être subdivisée en trois (au lieu de deux chez la femelle). Elle chevauche une bande d'activité phosphatasique acide exclusivement mâle.

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