STUDIES ON ENZYME ACTION.

XXXIII. Lipase Actions of Extracts of the Whole Rat at Different Ages.

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INTRODUCTION.

The difficulties encountered in following the chemical changes which occur in the development of a living organism have been emphasized frequently. The insistance of Jacques Loeb that definite chemical laws underlie such changes and may be discovered by suitable means of experiment, and the results which he obtained in such various lines of work, as a rule by the use of simple methods, encouraged the work of many who otherwise would not have entered the field. The present paper gives the results of a study of certain simple enzyme actions at different stages of growth of the whole rat. It is presented here as a contribution to the quantitative study of the changes which occur in life processes, a problem whose general significance and importance have been made clear by Jacques Loeb.

The lipase or ester-hydrolyzing actions of aqueous extracts of the whole rat at different ages covering the life cycle as far as possible from 3 days before birth until the age of 3 years 15 days will be presented. The reasons for selecting these enzyme actions were given in previous papers in which a number of the results obtained in the comparative study of such actions of tumors and normal tissues from various sources were given.[1]

[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183, 213; 1924–25, lxii, 687, 697; *J. Cancer Research*, 1925, ix, 105, 129.

EXPERIMENTAL METHODS AND RESULTS.

Albino rats, both male and female, were used. The ages of those which were obtained before birth were calculated on the assumption of a gestation period of 22 days and by comparison of their weights with those given by Donaldson[2] for rats under similar conditions. A number of the older rats were obtained from the Wistar Institute of Anatomy and Biology. All the rats which were used were normal as far as could be told. They were killed with ether, passed through a meat chopper twice, and water and toluene added at once. The hair was not removed as it had been found that it exerted no lipase action under the conditions used. The amount of water used was governed by the subsequent concentration of the solution desired for the enzyme tests. After standing overnight, the mixtures were filtered through paper, cloudy or turbid liquids being obtained. These were used for the enzyme tests, after being brought to pH 7.0 initially and suitably diluted. The conditions of testing the lipase actions were the same as those described previously; 15 cc. of solution, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator, duplicate and blank determinations, toluene present throughout.

Experimental Results.

The experimental results will be given only for rats whose ages are definitely known. The actions for various rats which may be classed as "adult" were determined, but since their ages were not known with any degree of exactness, these results will not be included here.

The absolute actions of the rat extracts on the different esters will not be given in detail here. A number of such values will be presented later in this paper (Fig. 4). The following data include the book number of the experiment (for reference); the ages of the rats in days, negative values referring to number of days before birth, zero ages to the fact that the rats were killed immediately after birth; and the

[2] Donaldson, H. H., The rat, Memoirs of the Wistar Institute of Anatomy and Biology, No. 6. Philadelphia, 2nd edition, 1924. *Cf.* also Ishii, O., *Anat. Rec.*, 1922, xxiii, 311.

weights of the rats in gm., or in the experiments with very young rats where more than one was used, the average weight.

Book No.....	122	115	126	106B	121	103	107	108	104
Age of rat, *days*...	−3	−2	−2	−1	−1	0	0	0	1
Weight " " *gm*....	2.0	2.4	2.7	3.5	4.2	4.1	4.4	4.9	4.9
Book No.....	120	119B	111	119A	110	140	139	133	109
Age of rat, *days*...	3½	4	5	8	11	12	17	22	22
Weight " " *gm*....	6.3	10.8	7.8	9.7	18.7	15.1	21.9	40.2	22.6
Book No.....	135B	137A	137B	136	129A	129B	113	130B	130A
Age of rat, *days*...	22	24	24	25	27	27	28	30	30
Weight " " *gm*....	27.8	26.0	28.6	34.2	24.1	26.5	50.2	26.4	28.6
Book No.....	134A	116(2)	116(1)	123	127	142	143	144	145
Age of rat, *days*...	30	40	40	71	89	100	126	189	285
Weight " " *gm*....	31.3	43.4	53.2	54.0	77.3	167.2	161.8	240.0	235.4
Book No.....	146	150	154	153	147	149	151	152	148
Age of rat, *days*...	367	545	555	740	748	913	994	1,055	1,110
Weight " " *gm*....	226.7	256.8	205.8	238.1	215.0	185.7	162.9	193.3	194.2

It is evident from the data that the weights of the rats did not increase entirely regularly with age. At times there were some striking irregularities (such as in Experiments 133 and 113). This may have been due, in part at any rate, to a smaller number of rats in the litter. As the rats became of more advanced age, they decreased in weight, though not in a regular manner. It may also be stated here that in 37 of the 45 experiments the concentrations of the extracts corresponded to 26.7 mg. of material per cc. of solution tested. In the other experiments the concentrations were somewhat larger.

As in the previous papers, two methods of comparing the actions present themselves; either as relative actions of a given extract on a number of esters, or as absolute actions. The first method has given the more interesting relations heretofore, although for a more satisfactory understanding of the results, both methods should be used. These two methods will also be followed in the present paper.

The relative actions on the esters are shown in Fig. 1. These are plotted with the action on phenyl acetate or methyl butyrate (whichever is the greater in the given experiment) as 100, and the actions on the remaining esters as percentages of this action. The points

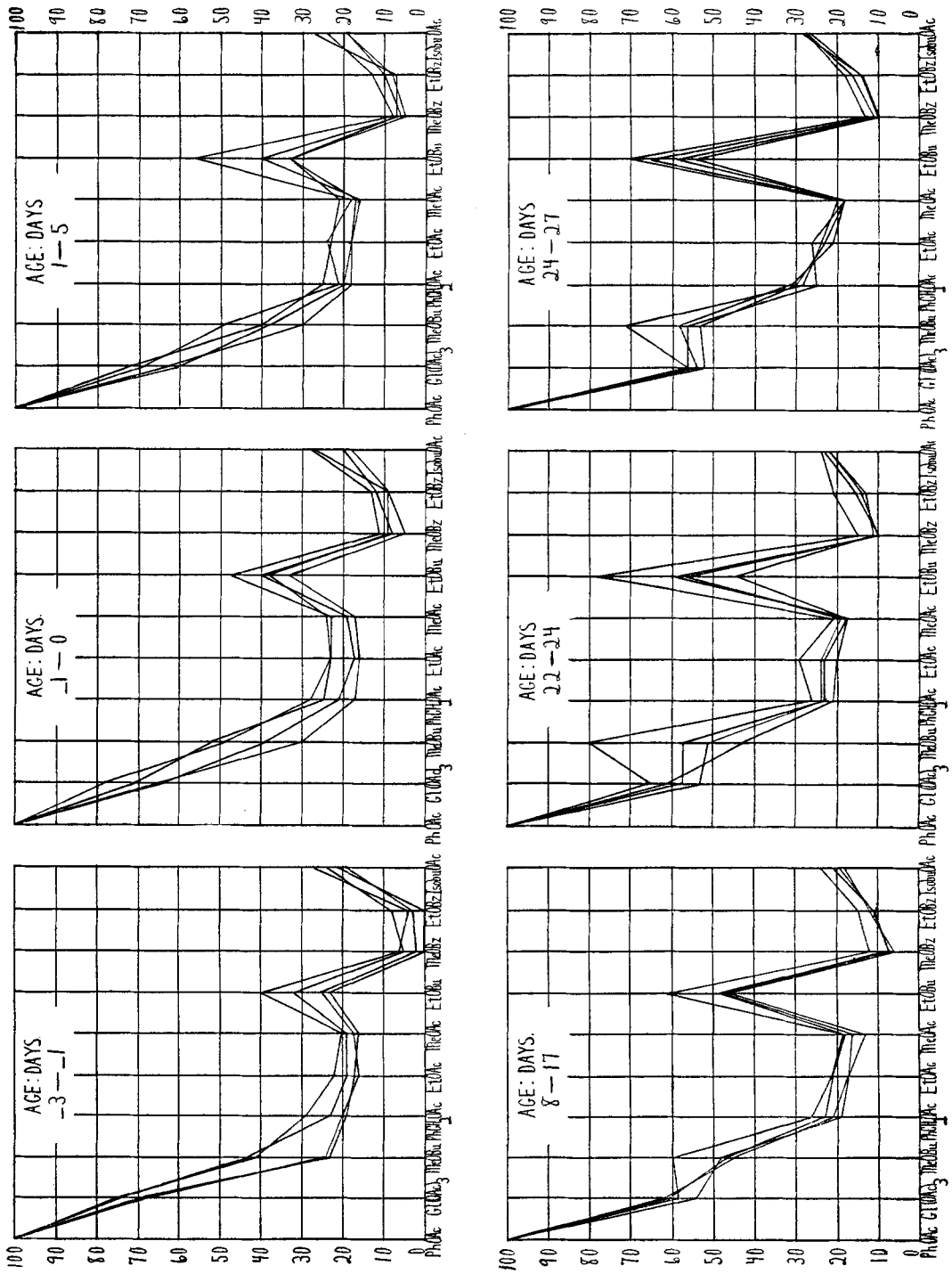
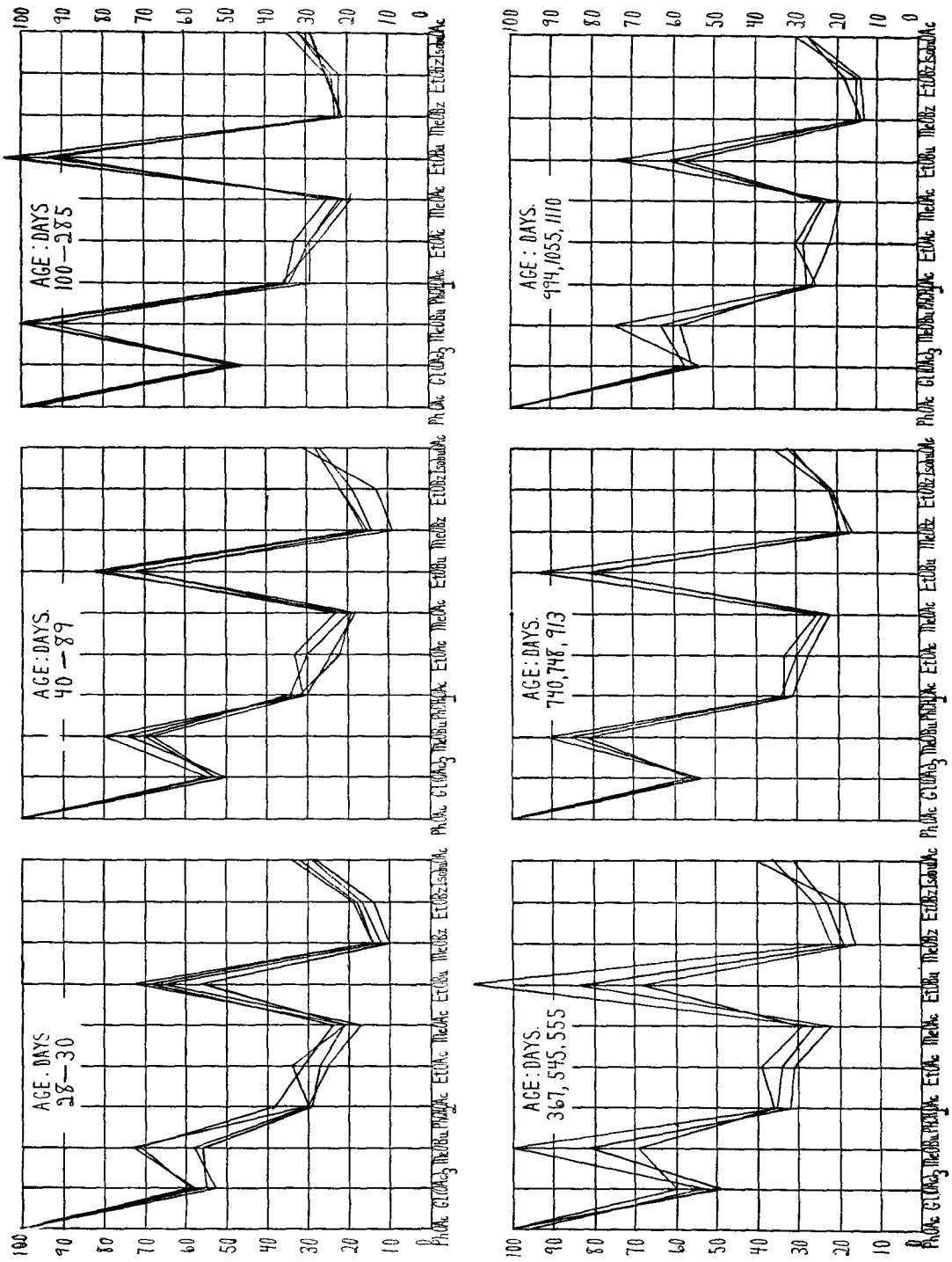


FIG. 1. Whole rats of different ages. Relative lipase actions. 45 curves, grouped in 12 plots, each curve representing the relative hydrolyzing actions on the esters indicated on the abscissa axes of the extract of a rat (or several rats) of definite age. The ages of the rats shown in each plot are



grouped without indicating the age of the rat for each curve. The general change in the "pictures" of the enzyme actions with increase in age of the rat is apparent, reaching a picture characteristic of the adult rat, and then appearing to revert again toward the embryonic type for the oldest rats.

denoting the actions are then connected. Each curve represents an experiment. The order in which the esters are arranged along the abscissa axis is the same as that used in the former papers and is a chance one.

There are 45 curves, one for each experiment. They are arranged four on a plot for the first 36 experiments, three on a plot for the last 9; twelve plots in all. The ages of the rats are not given for the separate curves, but indicated for the plot as a whole including the four or three curves as the case may be. The ages of the different rats, especially for the younger ones, are fairly close together, so that no sudden change in form of the curve occurs. Each plot represents therefore a group of rats with ages fairly close together and showing similar characteristics. For the oldest rats where some marked differences occur, differentiation of the rats is easy. The individual curves, therefore, were not labeled in the figure.

In studying the curves, certain definite regularities are apparent. Phenyl acetate action is placed at 100 in most of these comparisons. The most striking changes in the relative actions at the different ages are seen with the butyric esters. Starting at 20 to 40 per cent for the embryos, the relative values increase steadily to 50 to 60 per cent at 8 to 17 days. Somewhat wide variations, 50 to 80 per cent, are found at 22 to 24 days (weaning time), then a steady increase culminating at 100 per cent or over at 4 months to 1 year, with a decrease thereafter reaching 60 per cent at the age of 3 years. The actions on glyceryl triacetate showed only small variations. They started at 70 to 80 per cent, decreased gradually but steadily to about 50 per cent at the adult stage, and finally increased somewhat for the oldest rats. The relative actions on glyceryl triacetate and methyl butyrate are of interest; that on glyceryl triacetate being considerably larger in the earlier stages, the order being reversed after weaning, and tending to go back to the first order at old age. The benzoates started practically at 0 per cent, increased to more than 20 per cent, finally to decrease again.

Taking the actions of the esters in pairs, it will be seen that the ratios for the methyl and ethyl butyrates average very nearly 1.00, for the methyl and ethyl acetates very nearly 0.83, and for the methyl and ethyl benzoates very nearly 0.78. There are individual variations,

sometimes of considerable magnitude, but the differences from these averages are of such a character as to be evidently of experimental source. On the other hand, the ratios of the actions on the methyl esters to each other and of the ethyl esters to each other are markedly different, at the different ages. The ratio of methyl butyrate action to methyl acetate action is about 1.4 for the youngest rats, increases with considerable irregularity to a maximum of more than 5, and then decreases again to about 2.5. For the methyl acetate-methyl benzoate ratios, the values starting at 3 to 4 decrease to about 1 and then increase finally again. Because of some of the actions on methyl benzoate being very small with an accompanying large percentage experimental error, the ratios show very large irregularities at times. Similar results for the ratios are found in comparing the ethyl esters with different acid radicals.

These results indicate that the methyl and ethyl esters exert the same influence on the actions but that marked differences are shown when the acyl groups are changed with the alcohol radical (methyl or ethyl) constant. It may be pointed out that this first regularity appears to hold for the isobutyl radical as well since the actions on ethyl acetate and isobutyl acetate are very nearly the same throughout.

One more set of comparisons of this nature may be made; namely, that of the actions on the three sets of isomeric esters. The actions on phenyl acetate are much greater in every case than on methyl benzoate at the same time that the ratio of the actions reaches an apparent minimum of about five times as great values on the former as on the latter between the ages of 100 and 545 days. The action on benzyl acetate is much greater than that on ethyl benzoate for the embryos; thereafter it averages only about 50 per cent more. The action on ethyl butyrate is only slightly greater than on isobutyl acetate for the embryos and the first few days of life, thereafter the values for the actions on the former increase much more rapidly than for those on the latter with correspondingly larger values for the ratios until for the very oldest rats the ratios appear to decrease again.

Studying the curves of Fig. 1 as a whole, the regularity of the changes in relative enzyme actions with increase in age of the rats is striking. This regularity is not of mathematical nature in which each experiment falls into a definite place in the arrangement. There

are occasional irregularities in the curves at the different ages, the most marked of these irregularities being observed at and immediately after the time of weaning. It must be remembered in connection with such possible irregularities that individual living animals are being used, that a limited number of experiments (45) is being reported, and that every experiment in which the age of the rat was known definitely is given, no results being omitted for any reason whatever.

An interesting relation which follows directly from these curves and which may be of significance in connection with tumor growth, is that the embryonic rats approach the "picture" which was obtained with the Flexner-Jobling rat carcinoma[3] and with a considerable number of tumors of human origin.[4] As the rats grow older, the picture diverges more and more from this type, reaching apparently a characteristic picture or type of action for the adult rat and finally with the very old rats appearing to revert again and to approach the original young rat picture or type of action. These relations are brought out more clearly in Fig. 2 in which the curves are given for the rat at 2 days before birth (Experiment 2), and at the ages of 27 days, 126 days, and 1,110 days (Experiments 23, 34, and 45). The average of the Flexner-Jobling rat carcinoma actions as obtained previously is also given.

The possible influence of the presence of the young embryos as influencing the mother rat may be of interest. Fig. 3 shows the relative lipase actions of the mixture obtained from 9 embryo rats taken 1 day before birth as calculated from the gestation period, in comparison with the enzyme actions of the mother rat killed at the same time (Experiments 106A and 106B). The exact age of the mother rat was not known. The results indicate clearly however, that no influence was exerted on lipase actions of the mother rat as a whole under the given conditions.

A number of experiments were carried out on rats which carried the Flexner-Jobling rat carcinoma at different stages of growth. The rats were killed with ether, the tumor growths carefully removed

[3] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183.

[4] Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Cancer Research*, 1925, ix, 105.

(no metastases were observed) and the rats ground, extracted, and the extracts tested in the usual way. The curves for the relative actions

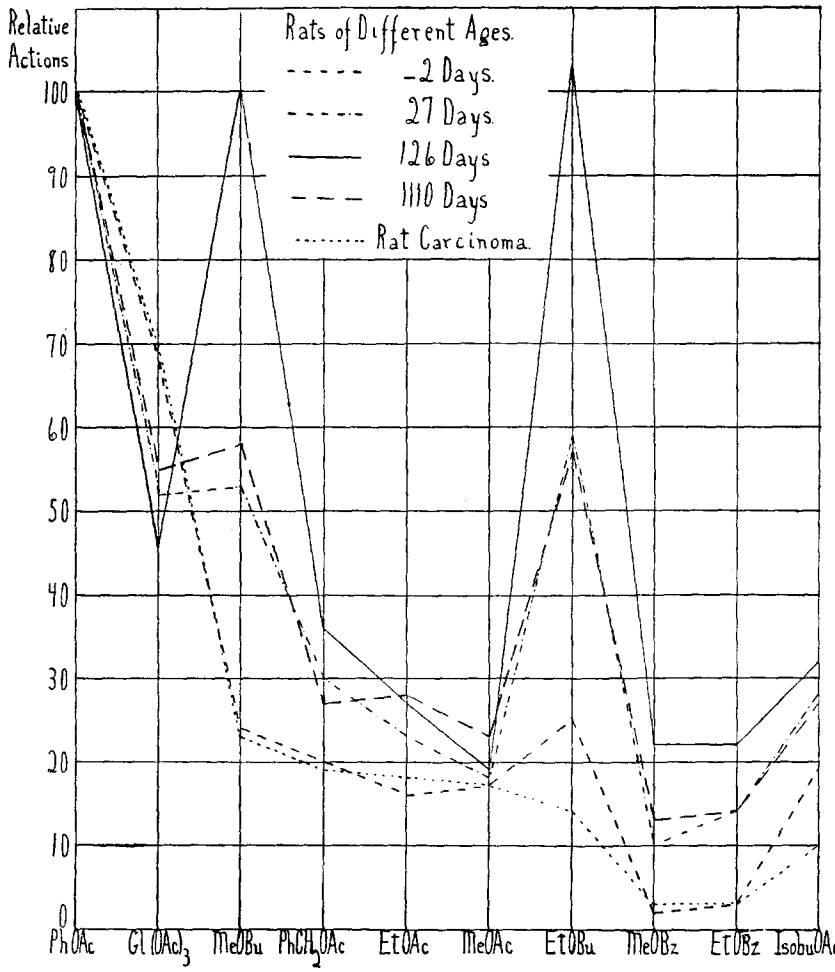


FIG. 2. Four rats of selected ages. Relative lipase actions. The curves of the lipase actions of four rats of definite ages are shown. The picture of the average results for the Flexner-Jobling rat carcinoma is also shown. The similarity of the rat embryo picture to the latter is apparent, as well as the change to the adult type and the seeming reversion in the direction of the embryonal type.

were found to fall into the positions on the plots which would be expected for rats which had no tumor growths. The figures indi-

cating these relations will, however, not be given, as they add nothing to the results already described.

In order to complete the presentation of the data as far as practicable a number of the absolute actions found with the different esters

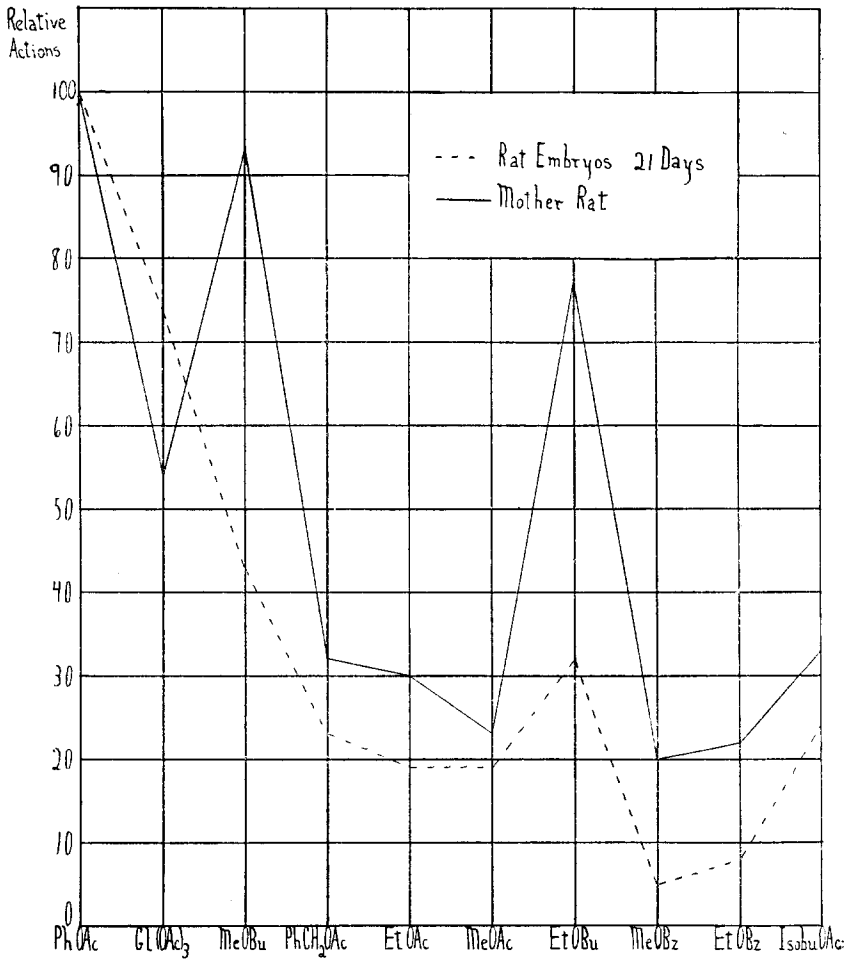


FIG. 3. Rat embryos (1 day before birth) and mother rat. Relative lipase actions. The differences between the two pictures is clear, as well as the lack of influence of the embryos on the picture of the lipase actions of the mother rat.

are shown in Fig. 4. Only those experiments are plotted for which the concentrations of the mixtures tested corresponded to 26.7 mg.

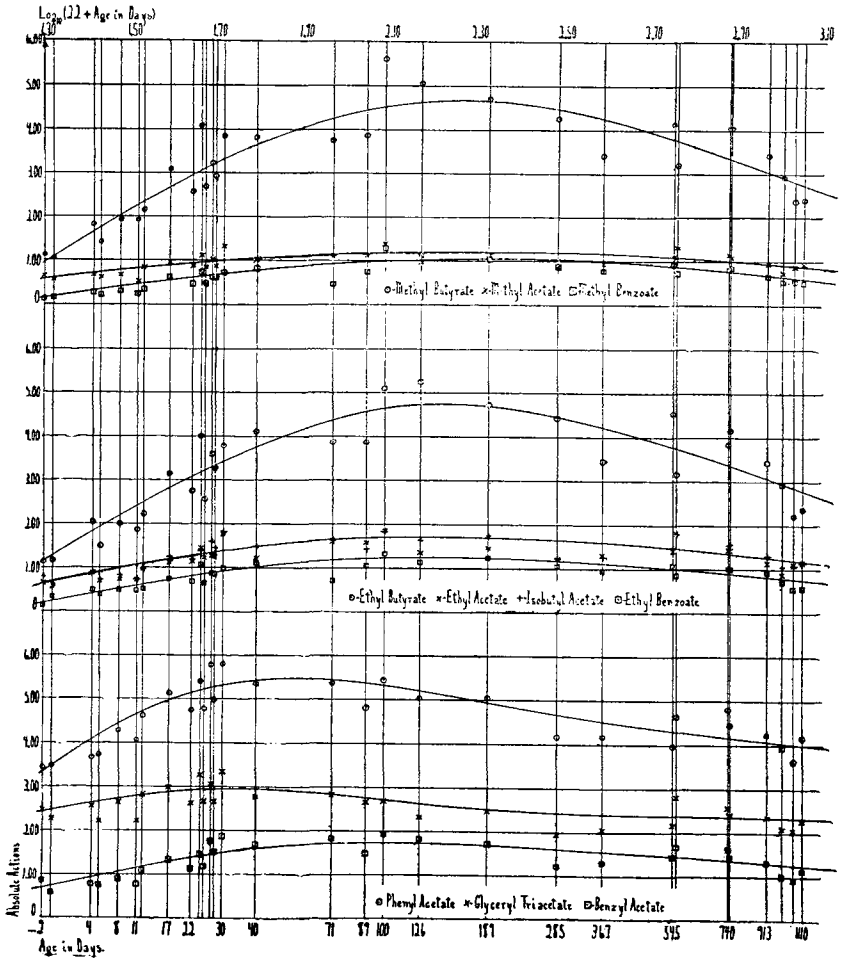


FIG. 4. Absolute ester-hydrolyzing actions of extracts of whole rats of definite ages at the same concentration. The curves are arranged in three groups. Absolute actions in tenths of milli-equivalents of esters hydrolyzed under the given conditions are plotted as ordinates; logarithms of the rat ages starting from day of conception are plotted as abscissas, $\log_{10}$ (22 + age of rat in days). The increases in actions for all the esters followed by decreases are evident, the amounts of the changes being different for the different esters and so giving rise to the different pictures shown in Figs. 1 and 2. Because of the method of plotting, the distances in the figure for the early part of the life of the rat are large and for the later periods very much condensed. The shapes of the curves are therefore distorted as far as showing the changes for equal intervals of time. For such equal intervals, the curves if plotted would show a steep initial rise or increase in actions followed by a very slow and gradual decrease.

per cc. of material originally extracted. The ages of the rats are plotted logarithmically on the abscissa axis, the zero point being taken as 22 days before birth (abscissa values are therefore $\log_{10}$ (22 + ages of rats in days). The absolute actions in tenths of milli-equivalents of ester hydrolyzed under the conditions of the experiments (cc. of 0.1 N sodium hydroxide used in the titrations) are plotted as ordinates. The actions for convenience are divided into three groups as shown.

There is considerable choice in the drawing of the curves for the actions on the different esters. This is evidently due to the variations in the individual rats and also possibly to a certain extent to the experimental method used. Even so, it is clear that any curves which may be drawn with the results as given would show the same general relation; that is, an increase in action from the embryo until a certain stage of maturity is attained, followed by a decrease. The absolute increases are different with different esters, greatest for the butyrates, smallest for glyceryl triacetate. The maximum appears to be different with the different esters, although here some uncertainty exists because of the lack of definiteness of the position of some of the curves. Farther than this it appears inadvisable to go at present with the results available.

The protease actions of 24 of the extracts of the whole rats of different ages were tested on a peptone preparation, a purified casein preparation, and gelatin. The methods used were similar to those described in an earlier paper,[3] 0.1 gm. of protein being used in each test, the mixtures brought to pH 7.0 initially, incubated at 38° for 22 hours, and the actions measured by the formol method.

The results obtained in these actions were not satisfactory in so far as the changes in action with increasing ages of the rats were concerned. It will not be necessary to give the detailed experiments, but only some of the most general conclusions. The actions on the peptone preparation appeared to be fairly constant throughout the life cycle of the rat, the irregularities which were observed being of such magnitudes as to be readily accounted for by the experimental inaccuracies. The actions on casein and on gelatin were much more irregular. No clear trends of changes in actions are evident. Several increases and decreases could be noted for each during the life cycle.

the most significant increases immediately after the rats were weaned. The other possible changes were too irregular and too uncertain to describe at present.

It was found in the earlier paper that the actions of isolated tissues of the rat gave distinctive actions on the peptone and the casein. Such different actions were not found so definitely for the whole rat at different ages. The natures of the two sets of experiments are different, but it is of interest to call attention to these results on the protease actions as compared with the lipase actions. The protease actions as measured refer to the chemical change involved in the hydrolysis of the peptide linking, but the measurements do not indicate the nature of the groupings combined with the carbon and nitrogen in the peptide linking. It is conceivable that at the different ages of the rats different peptide linkings are hydrolyzed but that the total amount of hydrolysis is not changed much. Under such circumstances, the protease actions would remain very nearly the same even with an entirely different part of the protein molecule hydrolyzed. The protease actions as measured measure, therefore, the amount of the action, but not necessarily the type of action. The limitation is not encountered in the ester-hydrolyzing or lipase actions as described where individual specific substrates are used and the measurement in each case shows not only the amount of the action but also its specific nature within the limits of the experiment. These considerations show the reason for the more satisfactory results obtained in the study of the enzyme actions with the ester-hydrolyzing actions rather than with the protein decompositions.

SUMMARY.

The ester-hydrolyzing or lipase actions of extracts of whole rats whose ages ranged from 3 days before birth to 3 years 15 days were tested on ten simple esters by the method described in previous papers. The results are presented in the form of curves, both as absolute actions and as relative actions on the different substrates. The "pictures" of the relative actions changed progressively with increasing age of the rat. For the embryo and the youngest rats, the curves approached those given by the Flexner-Jobling rat carcinoma and by a number of tumors of human origin, changing to a

type characteristic of the adult rat, and appearing to revert again to some extent to the embryonic type for the oldest rats. The changes in the actions on individual esters and the relative changes in the actions on different esters are discussed in detail. The greatest increases in actions as the rats became older were found with methyl and ethyl butyrates; at the same time that the actions on some of the other esters were also found to change in characteristic ways. Similar experiments with the protease actions of the extracts of whole rats of different ages on three protein preparations did not give differences similar to those found for the lipase actions. The probable reasons for these observed differences in the two sets of enzyme actions are discussed.