DISTRIBUTION AND PROBABLE MODE
OF PROLONGED ACTION OF POLY-OESTRADIOL
PHOSPHATE (PEP) IN SPAYED MICE

By

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I. INTRODUCTION

It has been reported previously that poly-oestradiol phosphate (hereafter PEP) a water soluble high molecular weight polyester of phosphoric acid and oestradiol-17β produces a greatly prolonged oestrogenic effect as measured by the mouse vaginal smear technique (Diczfalusy, 1954). This prolonged effect could be obtained following subcutaneous as well as intravenous administration. On the other hand, no oestrogenic effect could be demonstrated when PEP was administered orally.

At present the mechanism by which PEP elicits its greatly prolonged oestrogenic effect is not well understood. In order to find out, whether this prolonged action is associated with a depot formation in the organism, it seemed desirable to investigate the distribution of subcutaneously or intravenously administered PEP. For this purpose tracer technique appeared to be a suitable approach. Consequently, an attempt was made to study the distribution of radioactivity in different organs of spayed mice following the administration of PEP labelled with radioactive phosphorus (hereafter PE\textsuperscript{32}P).

In this paper data are presented indicating that subcutaneously or intravenously administered PEP does accumulate in certain organs of spayed mice. Additional data are also reported on the probable rôle of this accumulation in the mechanism by which PEP exerts its prolonged effect.

1. Presented before the Swedish Society of Endocrinology at a meeting held on December 3rd, 1954, in Stockholm.
II. MATERIAL

1. Preparation of PEP and PE$^{32}$P. - This will be reported elsewhere in detail (Fernö et al.). In essence the method consists of a condensation polymerisation reaction of oestradiol-17β with phosphorus oxychloride in dry pyridine at low temperature (approximately -10°C). The viscous reaction mixture is treated with crushed ice. The product formed is precipitated by dilute hydrochloric acid, dissolved in water at pH 8.0 and dialyzed against distilled water, until no inorganic phosphate is demonstrable in the solution. It is precipitated again and dried over phosphorus pentoxide. For the preparation of radioactive PEP (PE$^{32}$P), phosphorus oxychloride labelled with radioactive phosphorus ($^{32}$POCl$_3$) was used.2

2. Animals. - Adult female albino mice of the same colony have been used throughout the experiment. They were spayed at least 14 days before the administration of PEP. Prior to sacrifice a vaginal smear was taken from each animal in order to check that the animals sacrificed were still in oestrus.

III. METHODS

1. Design of experiments. - Groups of spayed mice were injected with PE$^{32}$P, or with the same amount of PEP, supplemented with a corresponding amount of radioactivity, in form of orthophosphate (Control-, or $^{32}$P-group). Groups of 20 animals were then killed from each series 1, 2, 4, 8 and 16 days following the administration of radioactive preparations, and the radioactivity present in the blood and various organs, measured.

2. Measurement of radioactivity. - All the radioactivity measurements have been carried out with one and the same thin-windowed Geiger-Müller counter (G.-M. tube).

Radioactivity data obtained with a particular G.-M. tube are subject to certain corrections depending i. a. on factors such as:
- resolving time of the tube
- background
- decay of radioactivity
- variation in efficiency of the tube
- variation due to sample geometry
- absorption losses in sample

These factors have been considered as follows:

Correction for resolving time. – A simplified correction formula,

2. The PEP- and PE$^{32}$P-preparations used in this study have been synthetized by the Research Laboratory of AB Leo, Hälsingborg, Sweden.
\[ C = \text{(counts)}^4/6000 \] was applied to all data. In this formula 6000 stands for the estimated maximal frequency (counts/min.) of the apparatus used, at which still reproducible values could be obtained. All experiments have been designed in such a way that the magnitude of correction should never exceed 5 per cent of the apparent counting rate. Under such conditions it is unlikely that the use of this correction formula will involve any major error.

**Correction for background.** In the present study measurement of radioactivity in various samples was only carried out by day and the background was estimated overnight. Thus each measurement of background is based on approximately 6000 counts. The extreme values of the background in 227 estimates during one year's period, were 6.2 and 9.7 counts/min. Therefore, it seems reasonable to conclude that the output of the G.-M. tube under the experimental period was satisfactorily constant. In view of the small variation of background values, it could be suggested that the use of a mean value will be more adequate than separate daily estimates of the background. In order to obtain information on this point, the error of G.-M. tube counting at the 6000-counts level was estimated as follows:

A standard of uranium oxide (\( \text{U}_3\text{O}_8 \)) with approximately 100 counts/min. was prepared. This standard was then counted 30 times consecutively, each time for 60 minutes. This counting gave a mean of 104.5 counts/min. with a variance per estimate = 9.92. The square root of this figure, \( \pm 3.15 \) counts/min. is the standard deviation (S. D.), characterizing the precision of our G.-M. tube counting at this level. From the pooled estimates of background obtained on 227 occasions, a mean value of 7.7 counts/min., with a variance of 0.264 was calculated. At this level (7.7 counts/min.) the variance depending on G.-M. tube counting would be 0.053. The ratio of variances (\( F = 0.264/0.053 = 4.9 \)) is significant on the 99.9 per cent probability level (\( n_1: 226, n_2: 29 \)). Thus it would appear that the day to day variation in background exceeds significantly the variation depending on G.-M. counting. Therefore it was considered to be better to use the background values obtained daily.

**Correction for decay of radioactivity.** All the radioactivity data presented in this study refer to the activity on the first day of experiment. They were calculated according to the general formula for decay of \( \text{P}^{32} \).

In order to ascertain that the radioactivity incorporated in our preparation consisted of \( \text{P}^{32} \), the decay of \( \text{P}^{32} \) was estimated experimentally, as shown in Fig. 1.

The value of the regression coefficient (0.021) shown in Fig. 1 can be proved to be identical with that obtained for the theoretical decay of a compound with a half-life time of 14.3 days, *i. e.* \( \text{P}^{32} \).

**Correction for variation in efficiency of the tube.** Any G.-M. tube may exhibit gross variation to a given radiation over a period of time. Thus in order to obtain reliable estimates of radioactivity, a suitable correction for this
Estimates of the decay of labelled poly-oestradiol phosphate (PE$_{32}$P). The equation of the regression line is $Y = 2.02 - 0.021 x$, where $y$ is measured in log counts and $x$ in days.

variation would seem to be essential. The magnitude of this correction is generally judged from the day to day response of the tube to a standard preparation. However, such a correction is only justified when this day to day variation in efficiency significantly exceeds the variation inherent in G.-M. tube counting (G.-M. tube counting error).

In this study, the variation in efficiency was checked by the use of a PE$_{32}$P standard preparation on each day when radioactivity measurements were carried out. Representative data obtained in one experiment are shown in Fig. 1. In order to obtain the day to day variance, the values obtained on different days were corrected for the decay of radioactive phosphorus.

Following correction, from the 37 estimates shown in Fig. 1 a mean value of 104.3 counts/min. with a variance per estimate = 17.2 can be obtained.

Since each of the 37 estimates shown in Fig. 1 is based only on 300 counts, the G.-M. tube counting error must also be estimated at the 300-counts level. Therefore the U$_3$O$_8$ standard previously used for the estimation of G.-M. tube counting error at the 6000-counts level, was counted again 30 times, on each occasion for 3 minutes. This counting gave a mean of 104.7 counts/min., with a variance of 26.1. The ratio of variances ($F = 26.1/17.2 = 1.52$) is not significant. Thus the day to day variation in efficiency does not exceed the G.-M. tube counting error.

It should be pointed out that the data of Fig. 1 embrace only a 48-days
period. A similar check on the efficiency was done, however, in each series of the experiment, with similar results. It is therefore concluded that – under the experimental conditions of this laboratory – the variation in efficiency of the G.-M. tube is well within the variation of G.-M. tube counting. Consequently, this factor was neglected in the course of this study. These results also indicate that under our experimental conditions it would not be justifiable to relay on daily single estimates of a standard $^{32}$P preparation, when correcting for decay.

**Correction for variation due to sample geometry.** – This error depends on the inhomogenous spreading of samples on the backing material. Since with a high energy emitter, such as $^{32}$P, this displacement effect can hardly be detected, this factor was entirely neglected.

**Correction for absorption losses in sample.** – This important source of error will be discussed separately in the next chapter under the heading »Preparation of organs for radioactivity measurements«.

3. Preparation of organs for radioactivity measurements. - In preliminary experiments a wet ashing technique was used; the organs were digested with concentrated sulphuric acid, the solution neutralized and the inorganic phosphate precipitated in form of magnesium ammonium phosphate. The precipitate formed was filtered by suction to small aluminium plates, according to the technique described by Lindberg (1946), dried and the radioactivity estimated. Since, however, this technique is very time-consuming, attempts have been made to replace wet ashing by a rapid dry ashing technique as follows:

The organs were placed in crucibles containing 0.9 gm. of magnesium nitrate and 0.5 gm. of sodium phosphate. The crucibles were heated in an electric oven at approximately 500$^\circ$C for 1 to 2 hours. They were allowed to cool and the contents of the crucibles were directly transferred to small aluminium discs. The crucibles were then rinsed with a small volume of carrier phosphate solution and the solution also transferred to the aluminium plate. Three drops of a saturated shellac solution in ethanol were added and the contents of the discs dried at room temperature.

In order to assess the precision of the dry ashing and wet ashing techniques respectively, and furthermore, in order to measure the magnitude of losses arising from the preparation of samples as well as from absorption in the samples, the experiment shown in Table 1 was carried out.

The »without treatment«-group was prepared by pipetting labelled phosphate directly into the discs and by drying them at room temperature following the addition of 3 drops of shellac solution. It would appear from the data shown in Table 1, that a significant loss in radioactivity occurs, when labelled phosphate is carried through the one or the other ashing procedure. The difference between the values obtained without treatment on the one hand and those obtained following wet ashing and dry ashing respectively on the other hand, are signi-
Recovery of radioactive phosphate added to different organs and submitted to various ashing procedures.

Figures indicate mean counts/min. ± standard deviation of a single estimation (S.D.).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Number of estimates (n)</th>
<th>Mode of preparation</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without ashing</td>
<td>Wet ashing + filtration</td>
<td>Dry ashing</td>
<td></td>
</tr>
<tr>
<td>Without organ</td>
<td>16</td>
<td>130.3 ± 4.5</td>
<td>111.0 ± 7.9</td>
<td>112.1 ± 13.7</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>16</td>
<td>–</td>
<td>105.5 ± 5.5</td>
<td>93.6 ± 12.5</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>16</td>
<td>–</td>
<td>107.4 ± 8.3</td>
<td>95.4 ± 13.0</td>
<td></td>
</tr>
</tbody>
</table>

Significant on the 99.9 per cent probability level. It is also apparent from Table 1 that the use of the dry ashing method results in a significant loss in precision as compared with the wet ashing technique. On the other hand, no difference is encountered in the use of the same method on various organs. It seems therefore justifiable to assume that radioactivity data obtained with the same method on different organs are comparable.

When the values obtained following dry ashing in the presence of liver and brain respectively, are combined, a mean recovery of 73 per cent can be calculated, as it would appear from Table 1. Thus the use of the dry ashing technique involves a loss in radioactivity of approximately 27 per cent. Consequently all radioactivity data obtained following dry ashing of different organs have been corrected for this loss. A similar loss of approximately 18 per cent can be calculated from the data obtained following wet ashing.

Thus the loss in radioactivity due to ashing may be an important source of error, whenever counts measured in various organs are expressed as percentages of the administered dose.

Throughout this study the dry ashing method was used, since it was assumed that the increment in error depending on the use of dry- instead of wet ashing, will almost be negligible when compared with that depending on biological variation and processing. That this assumption is justifiable, would appear from the data shown in Table 2, where the variances due to different ashing techniques used in the preparation of organs for radioactivity measurements, are compared with those actually found in various organs in the course of the experiment.

Blood samples were not carried through the ashing procedure, but were dried and read directly. Thus it was necessary to examine whether or not the presence of 0.2 ml. blood on the discs will lead to absorption losses. Therefore labelled phosphate was pipetted onto 30 discs. To 15 of these an additional volume of
Table 2.
Comparison of variances calculated from estimates of radioactivity in different organs with those depending on various ashing techniques used in the preparation of organs.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Organ</th>
<th>Mode of preparation</th>
<th>Degrees of freedom (d. f.)</th>
<th>Counts/min.</th>
<th>Variance per estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td>-</td>
<td>Dry ashing</td>
<td>45</td>
<td>100.4</td>
<td>157.9</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Wet ashing</td>
<td>45</td>
<td>108.0</td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>Dry ashing</td>
<td>18</td>
<td>94.0</td>
<td>842.8</td>
</tr>
<tr>
<td></td>
<td>» Liver</td>
<td>»</td>
<td>14</td>
<td>109.3</td>
<td>874.2</td>
</tr>
<tr>
<td></td>
<td>» Skin</td>
<td>»</td>
<td>17</td>
<td>79.3</td>
<td>1063.6</td>
</tr>
<tr>
<td></td>
<td>Residual fraction</td>
<td>»</td>
<td>19</td>
<td>97.0</td>
<td>4421.3</td>
</tr>
</tbody>
</table>

0.2 ml. heparinized blood was added. The samples were dried at room temperature and counted. The control group gave 81.5 ± 4.44 (S. D.) counts/min., whereas the plates covered with 0.2 ml. blood gave 82.0 ± 6.19 counts. In view of this evidence, no correction for absorption losses was applied to radioactivity data in blood.

IV. STATISTICAL METHODS

1. Linearisation of radioactivity-curves. - In order to facilitate the comparison of radioactivity data obtained on various organs following different treatments, it is advantageous to obtain a linear relationship between counts and time of experiment. This can generally be achieved by the use of transformations. By transforming the radioactivity curves into straight lines, it becomes possible to assess the significance of differences in the amount of radioactivity incorporated into the various organs throughout the whole experiment as well as to compare adequately the rate of disappearance of radioactivity from different organs. The most frequently used procedure in radioactivity studies is to transform the dependent variable (counts/min.) into logarithms. An example for the use of transformations is shown in Fig. 2, where the mean counts per ml. blood on different days following the subcutaneous administration of PE³²P and ³²P respectively, are presented in different forms.

Fig. 2 A shows the curves obtained by plotting counts/ml. blood against time elapsing between injection and sacrifice of animals. Apparently, it is rather
The influence of various transformations on the shape of radioactivity curves obtained in the blood of spayed mice following a single subcutaneous injection of labelled polyoestradiol phosphate (PE$^{32}$P = filled circles) and of labelled inorganic phosphate ($^{32}$P = open circles), respectively.

In Fig. 2 A counts/min. are plotted against days; in Fig. 2 B log counts/min. against days, and in Fig. 2 C log counts/min. against the cubic root of days. The dotted lines in Fig. 2 B indicate that the use of this transformation led to significant evidence against the hypothesis of linearity.

difficult to compare the disappearance rate of the two preparations from this figure. When the logarithms of counts are plotted against days, the lines shown in Fig. 2 B can be obtained. Since, however, statistical analysis indicated that the lines shown in Fig. 2 B are invalid, attempts have also been made to transform the independent variable. Several transformations seemed to yield valid lines. When for instance the following transformation, $x = t^{1/3}$ (where $t$ is the time measured in days) was applied to the data of Fig. 2 B, statistically valid lines were obtained. These lines are shown in Fig. 2 C.

In order to illustrate the procedure employed in the calculation of the lines and in the assessment of their validity, an example is given in Tables 3 and 4.

Table 3 summarizes the blood data found in $^{32}$P-treated animals (shown with open circles in Figs. 2 B and 2 C).

The analysis of variance for the data of Table 3 will be considered on ground of two hypotheses, namely, 1) that the true relationship between days and log counts is linear (as shown in Fig. 2 B) and 2) that the true relationship between the cubic root of days and log counts is linear (as shown in Fig. 2 C).
Table 3.
Disappearance of radioactivity from the blood of spayed mice following a single subcutaneous injection of radioactive phosphate ($^{32}$P).
Radioactivity is expressed as log counts/min. per ml. blood.

<table>
<thead>
<tr>
<th>Days</th>
<th>n</th>
<th>Sums of log counts</th>
<th>Mean log counts ± S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>38.953</td>
<td>1.948 ± 0.079</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>33.717</td>
<td>1.775 ± 0.105</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>26.125</td>
<td>1.587 ± 0.112</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>23.234</td>
<td>1.223 ± 0.143</td>
</tr>
<tr>
<td>Totals</td>
<td>75</td>
<td>122.029</td>
<td>—</td>
</tr>
</tbody>
</table>

The equation of the line connecting the points is given by the usual equation of a regression line

\[ Y = \bar{y} + b (x - \bar{x}) \]  (1)

where \( \bar{y} \) indicates mean log counts calculated from all the data, \( \bar{x} \) is mean time in days (assumption 1) or in cubic root of days (assumption 2) and \( b \) is the regression coefficient or slope calculated from the formula

\[ b = \frac{S_{xy}}{S_{xx}} \]  (2)

where \( S_{xy} \) indicates the sum of products of deviations of \( x \) and \( y \), i.e. \( S(x - \bar{x}) (y - \bar{y}) \), and \( S_{xx} \) is the sum of squares of deviations of \( x \), i.e. \( S(x - \bar{x})^2 \).

Implicit in the use of this formula of \( b \) is the assumption that the variance of \( y \) is approximately constant for all values of \( x \). It is therefore necessary to check on the homogeneity of variances. This has been carried out by applying Bartlett's test (1937) to the data. Since on no occasion was it possible to obtain significant evidence of heterogeneity, the application of regression equations to the data of the present study appeared justifiable.

Thus the equation of the $^{32}$P-line can be estimated as $Y = 1.627 - 0.100 (x - 3.706)$ and $Y = 1.627 - 0.726 (x - 1.453)$ respectively, which after simplification is reduced to $Y = 1.998 - 0.100 x$ (assumption 1) and $Y = 2.682 - 0.726 x$ (assumption 2).

The analysis of variance for the data of Table 3 is shown in Table 4.

The total sum of squares of deviations is formed from all the 75 individual data. This total sum of squares is partitioned then into two components »between days« (with 3 degrees of freedom) and »within days« (with 71 d.f.).

3. The reader interested in details of computation is referred to textbooks on statistics, such as those by Goulden (1952), or by Snedecor (1955).
Table 4.
Analysis of variance for the data of Table 3 calculated on different assumptions about the independent variable.

<table>
<thead>
<tr>
<th>Transformation</th>
<th>Assumption 1</th>
<th>Assumption 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x = t (days)</td>
<td>x = t'/6</td>
</tr>
<tr>
<td></td>
<td>y = log counts/min.</td>
<td>y = log counts/min.</td>
</tr>
<tr>
<td>Adjustment for mean</td>
<td>198.548</td>
<td>198.548</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>Sum of squares</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>5.577</td>
<td>0.135</td>
<td>5.708</td>
<td>0.004</td>
</tr>
<tr>
<td>Deviation from regression</td>
<td>2</td>
<td>0.135</td>
<td>0.068</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Between days</td>
<td>3</td>
<td>5.712</td>
<td>0.887</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>Error (within days)</td>
<td>71</td>
<td>6.599</td>
<td>0.012</td>
<td>6.599</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>6.599</td>
<td></td>
<td>6.599</td>
<td></td>
</tr>
</tbody>
</table>

The »between days«-component can further be subdivided into a separate d.f. for regression (calculated as \((S_{xy})^2 / S_{xx}\)), and the remainder which is associated with 2 d.f. will represent the scatter around the line, i.e. deviations from regression. By dividing the sum of squares with the corresponding d.f., the mean squares can be obtained, as indicated in separate columns.

A test of significance of evidence against the hypothesis of linearity may be obtained by testing whether the mean square depending on deviations from linearity (deviations from regression) significantly exceeds the error mean square (within days-component). This can be checked by calculating F, the ratio of the first mean square to the second. These F-values are then compared with the tabulated 5 per cent probability values for the distribution of variance ratio (Fisher & Yates, 1953), using the entry corresponding to the number of d.f. associated with the two mean squares respectively. In the present case F calculated from the data obtained with assumption 1 is estimated as F = 0.068, 0.012 = 5.67, which greatly exceeds the allowable limit (3.10). Therefore the evidence obtained against linearity must be considered as significant. Consequently, the assumption that the relationship between log counts and days is linear is untenable and must be rejected.

On the other hand, from the data calculated with assumption 2, no significant
evidence of non-linearity can be obtained. Thus the hypothesis that the relationship between log counts and cubic root days is linear, does not seem to be contradicted by the experimental data. Consequently, in this case, the use of the cubic transformation would appear to be justified.

In order to find out which transformation of the independent variable will result in valid lines in the majority of cases, radioactivity values obtained in different organs (log counts/ml. blood and log counts/gm. tissue, respectively) have been analyzed on different assumptions about $x$, in the same way as indicated in Table 4. The $F$-values obtained together with the allowable limits are compiled in Table 5.

The last two columns of Table 5: log $(t + 1)$ and $(t + 0.75)^{1/3}$ have been derived from the general forms log $(t + c)$ and $(t + c)^{1/3}$, respectively, by assigning various values to the constant $c$, and checking graphically the goodness of fit of the resulting lines, as recommended by Moore & Tukey (1954).

It would appear from the data shown in Table 5, that the best results can be expected by the use of one of the following transformations: $(t + 0.75)^{1/3}$, $t^{1/2}$ or $t^{1/4}$.

2. Adjustment for differences in organ weight. - In the calculation of the variance ratios shown in Table 5, the radioactivity data have been expressed as log counts/gm. organ weight. The expression of results in this form involves the arbitrary assumption that the concentration of radioactivity in various organs is in direct proportion to the organ weight. In the course of preliminary computations it was found that adjustment by covariance analysis instead of proportionality, may be more effective in improving the precision of the estimate of regression lines. An example is shown in Fig. 3, where the logarithms of counts found in the liver of PE$^{32}$P-treated animals are plotted against the cubic root of time in days, A) without adjustment for differences in organ weight (i.e. log counts/liver), B) following adjustment by proportionality (i.e. log counts/gm. liver) and C) after adjustment for differences in organ weight by covariance analysis.

The data presented in Fig. 3 seem to indicate that in this case the extra work involved in covariance analysis may be justified, since the precision of the line is apparently improved. Since, however, the gain in precision due to covariance analysis may vary greatly from experiment to experiment and also from organ to organ, the decision about the ideal policy can not be based upon analysis of a single experiment. Consequently, data obtained on different organs following treatment with PE$^{32}$P and $^{32}$P respectively, were analyzed a) without

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4. The technique of covariance analysis will not be discussed here; for details of computation the reader is referred to the textbooks by Goulden (1952), Finney (1952) and by Snedecor (1955).
Table 5.

Validity of regression lines judged from the ratio of variances (F) depending on scatter about the regression line to that due to sampling error. Data calculated on different assumptions about the independent variable.

In this table $t$ is the time in days elapsing between the start of experiment and sacrifice of animals, $n_1$ stands for the number of degrees of freedom (d. f.) used in calculating the variance due to deviations from regression (scatter about the line), $n_2$ is the number of d. f. associated with sampling error (within days variation) and limit stands for the 5 per cent probability value for the distribution of variance ratio (Fischer & Yates, 1953) with the indicated values of $n_1$ and $n_2$. F-values exceeding this limit indicate significant evidence of non-linearity.

If $F$ is less than 1.0, its reciprocal must be entered in the table of variance ratio, with $n_1$ and $n_2$ interchanged.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment</th>
<th>Route of administration</th>
<th>$n_1$</th>
<th>$n_2$</th>
<th>Limit ($p = 0.95$)</th>
<th>Values of variance ratio (F) calculated by assigning values to the independent variate as</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t$</td>
<td>$t^{1/2}$</td>
</tr>
<tr>
<td>Blood</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>2</td>
<td>71</td>
<td>3.13</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>2</td>
<td>70</td>
<td>3.13</td>
<td>3.58</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>i. v.</td>
<td>2</td>
<td>75</td>
<td>3.12</td>
<td>19.43</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>i. v.</td>
<td>2</td>
<td>75</td>
<td>3.12</td>
<td>25.60</td>
</tr>
<tr>
<td>Liver</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>86</td>
<td>2.73</td>
<td>42.70</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>80</td>
<td>2.74</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>i. v.</td>
<td>3</td>
<td>90</td>
<td>2.73</td>
<td>7.30</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>i. v.</td>
<td>3</td>
<td>87</td>
<td>2.73</td>
<td>1.70</td>
</tr>
<tr>
<td>Kidney</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>71</td>
<td>2.75</td>
<td>16.80</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>55</td>
<td>2.75</td>
<td>2.49</td>
</tr>
<tr>
<td>Skin</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>87</td>
<td>2.73</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>84</td>
<td>2.73</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Counts/ min.  

**Fig. 3.**

Influence of various adjustments for differences in organ weight on the precision of a line, representing the disappearance of radioactivity from the liver of spayed mice following a single subcutaneous injection of labelled poly-oestradiol phosphate (PE\(^{32}\)P). The lines have been obtained by plotting log counts/min. against the cubic root of time (in days). For the sake of convenience, both the logarithmic and cubic root scales are graduated in true units.

adjustment for organ differences, b) following adjustment by proportionality and c) after adjustment by covariance analysis. The mean squares depending on deviations from regression (\(s_1^2\)) and on sampling error (\(s_0^2\)) are compiled in Table 6.

It should be noted that the data shown in Table 6 have been computed on the assumption that the relationship between log counts and cubic root of time in days is linear.

Perusal of the data shown in Table 6 reveals that both adjustment by proportionality and by covariance analysis greatly improve the precision. Therefore the neglect of any adjustment for differences in organ weights under our experimental conditions would be unjustifiable. It is also apparent from Table 6, that the gain in precision due to covariance analysis over that obtained by assuming proportionality, is very slight, if any. Consequently, throughout the whole study radioactivity data will be corrected for differences in organ weight by assuming proportionality and will be expressed as log counts/gm. organ.

3. Final choice of transformation and adjustment. - In view of the evidence presented in this chapter, it was decided that for the purposes of the present investigation the following transformations and adjustments should be used: 
\[
x = (t + 0.75)^{1/2}
\]
as the independent variable, where \(t\) is measured in days, and 
\[
y = \log \text{counts/min.}
\]
as dependent variable, where counts are adjusted for differences in organ weight by proportionality and expressed as log counts/min. per gm. organ.

The choice of \(x = (t + 75)^{1/2}\) as a transformation for the independent variable was thought to be motivated by the fact that its use resulted in most cases in valid lines, with relatively small scatter of experimental points.
Table 6.
Influence of various adjustments for differences in organ weight on the precision of estimates of radioactivity in different organs. In this table $s_1^2$ indicates the mean square depending on deviations from linearity, $s_2^2$ is the mean square due to sampling error, and $n_1$ and $n_2$ stand for the number of degrees of freedom associated with $s_1^2$ and $s_2^2$ respectively.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment</th>
<th>Route of administration</th>
<th>No adjustment</th>
<th>Proportionally</th>
<th>Covariance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$n_1$</td>
<td>$n_2$</td>
<td>$s_1^2$</td>
</tr>
<tr>
<td>Liver</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>87</td>
<td>0.134</td>
</tr>
<tr>
<td></td>
<td>PE$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>81</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>$^{32}$P</td>
<td>i. v.</td>
<td>3</td>
<td>91</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>PE$^{32}$P</td>
<td>i. v.</td>
<td>3</td>
<td>88</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>PE$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>70</td>
<td>0.144</td>
</tr>
<tr>
<td>Kidney</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>71</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>PE$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>54</td>
<td>0.154</td>
</tr>
<tr>
<td>Skin</td>
<td>$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>87</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>PE$^{32}$P</td>
<td>s. c.</td>
<td>3</td>
<td>84</td>
<td>0.448</td>
</tr>
</tbody>
</table>
It should be noted that in all figures to be presented the scales used will correspond to these transformations, but – for the sake of convenience – they will be graduated in true units.

V. RESULTS

1. Distribution of radioactivity following subcutaneous administration of PE32P. - In this experiment 100 spayed mice were injected subcutaneously with PE32P in direct proportion to their body weight; an average mouse of 20 gm. being injected with 45 µg. of PE32P corresponding to 7716 ± 623 counts/min. (mean ± S.D., based on 20 estimates). A control group of 100 spayed mice was injected with the same amount of non-labelled PEP supplemented with 7848 ± 306 counts/min. (n = 20) in form of orthophosphate (32P). Since the difference between the radioactivity doses administered to different groups is not significant, it seems justifiable to conclude that experimental- and control-groups were injected with the »same« amount of radioactivity.

On the 1, 2, 4, 8, and 16th day of experiment, groups of 20 PE32P-, and 32P- treated animals respectively, were anesthesized with chloroform and the thorax opened. 0.2 ml. blood was withdrawn with a heparinized pipette and plated for radioactivity measurement. The organs to be analyzed were rapidly dissected out and weighed on a Mettler automatic balance to the nearest 0.1 mg.

The data obtained on the analysis of radioactivity in the different organs are summarized in form of regression lines. The regression coefficients (b) ± their standard errors (sb) estimated from the data obtained, are compiled in Table 7. The regression coefficients (b) were estimated as shown in formula (2) (page 13), whereas the standard error of b, sb was calculated as

\[ s_b = \sqrt{\frac{S_{yy} - \frac{(S_{xy})^2}{S_{xx}}}{(n - 2) S_{xx}}} \]  

(3)

where Sxy and Sxx have the same meanings as in formula (2), and Syy = S (y - \overline{y})², i. e. the total sum of squares of deviations of y.

Radioactivity of the following organs was measured:

- skin at the site of injection
- blood
- liver
- spleen
- uterus
- kidney
- brain
- residual fraction.
Skin at the site of injection. – The skin and subcutaneous connective tissue were removed from the site of injection, excising an area of approximately 4 cm². The lines computed from the data obtained PE³²P- and ³²P-treated groups respectively, are shown in Fig. 4.

The differences between the two slopes is not significant (see Table 7), indicating that the radioactivity administered as PE³²P, or as ³²P, disappears with approximately the same rate from the site of injection. It is also apparent from Fig. 4 that the amount of radioactivity is much higher in the skin of PE³²P-treated animals than in that of ³²P-injected ones. In fact the difference between ŷ-values is highly significant (p < 0.001). Consequently, the conclusion that there is a subcutaneous PE³²P-depot seems to be justified. However, the amount recoverable from the skin at the site of injection 24 hours following the administration of PE³²P does not exceed 10 per cent of the dose administered. This fact indicates that approximately 90 per cent of the radioactivity administered as PE³²P disappears from the site of injection within 24 hours.

![Image of Fig. 4](image-url)

Disappearance of radioactivity from the skin at the site of injection in spayed mice following a single subcutaneous injection of labelled poly-oestradiol phosphate, PE³²P (filled circles) and of labelled inorganic phosphate, ³²P (open circles), respectively. The dose administered to the PE³²P-treated animals was 7716 counts/min. per 20 gm. body weight and that injected into the ³²P-treated animals, 7848 counts/min. per 20 gm. body weight. The equation of the PE³²P-line is Y = 3.249 - 0.158 x, and that of the ³²P-line Y = 2.323 - 0.228 x. The scale of the abscissa corresponds to \( (t + 0.75)^{1/2} \), where \( t \) is measured in days, whereas the scale of the ordinate is logarithmic. In this and in all following figures these scales will be used, but for the sake of convenience they will be graduated in true units.
In our attempt to remove the whole site of injection, a rather large piece of skin was extirpated from each animal. The weight of these pieces varied considerably. Assuming now, that the radioactivity injected as PE\textsuperscript{32P} is concentrated only within a small area of the extirpated piece of skin, it would seem reasonable to expect that radioactivity data unadjusted for differences in skin weight will provide more accurate estimates than those based on various adjustments. However, the data presented in Table 6 do not seem to verify this assumption; on the contrary, it appears from the data of Table 6 that adjustment of the skin-data by proportionality or by covariance analysis will greatly improve the precision of the lines calculated. Under such circumstances it was felt justifiable to express the radioactivity data as log counts/gm. skin.

Blood. – The radioactivity data obtained in the blood are presented in Fig. 5.

Table 7.
Values of regression coefficients, indicating the disappearance rate of radioactivity from different organs of spayed mice. PE\textsuperscript{32P} indicates animals treated with labelled poly-oestradiol phosphate; \textsuperscript{32P} indicates mice treated with the same amount of non-labelled poly-oestradiol phosphate plus labelled inorganic phosphate.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment</th>
<th>Regression coefficient (b ± s\textsubscript{b})</th>
<th>Significance of the difference between regression coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PE\textsuperscript{32P}</td>
<td>\textsuperscript{32P}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PE\textsuperscript{32P}</td>
<td>PE\textsuperscript{32P}</td>
</tr>
<tr>
<td>Skin</td>
<td>a</td>
<td>-0.158 ± 0.022</td>
<td>-0.228 ± 0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Blood</td>
<td>a</td>
<td>-0.435 ± 0.020</td>
<td>-0.440 ± 0.020</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-0.457 ± 0.013</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>-0.568 ± 0.072</td>
<td>-0.276 ± 0.080</td>
</tr>
<tr>
<td>Liver</td>
<td>a</td>
<td>-0.056 ± 0.018</td>
<td>-0.348 ± 0.016</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-0.103 ± 0.011</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>-0.096 ± 0.035</td>
<td>-0.317 ± 0.045</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td>a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.005 ± 0.017</td>
<td>-</td>
</tr>
<tr>
<td>Uterus</td>
<td>a</td>
<td>-0.368 ± 0.027</td>
<td>-0.274 ± 0.042</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Kidney</td>
<td>a</td>
<td>-0.364 ± 0.023</td>
<td>-0.316 ± 0.017</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Brain</td>
<td>a</td>
<td>0.157 ± 0.070</td>
<td>0.128 ± 0.058</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Residual fraction</td>
<td>a</td>
<td>-0.035 ± 0.014</td>
<td>-0.089 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-0.024 ± 0.014</td>
<td>-0.052 ± 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a)} Animals treated subcutaneously.  
\textsuperscript{b)} Animals treated intravenously.  
\textsuperscript{c)} Animals treated subcutaneously. Analyses carried out following extraction of organs with trichloroacetic acid and ethanol-ether.
Fig. 5. Disappearance of radioactivity from the blood of PE$^{32}$P-treated (filled circles) and $^{32}$P-treated (open circles) animals. Route of administration: subcutaneous. The equation of the PE$^{32}$P-line is $Y = 2.977 - 0.435x$, and that of the $^{32}$P-line is $Y = 2.374 - 0.440x$.

For further particulars consult Fig. 4.

Since it has not been possible to measure on the 16th day of experiment the low radioactivity in the blood of $^{32}$P-treated animals, Fig. 5 shows only the data obtained on days 1–8.

It follows from Table 7, that the two regression lines are parallel, indicating that the radioactivity is cleared with the same rate from the blood of PE$^{32}$P- and $^{32}$P-injected animals, respectively. Similarly to the situation observed in the skin, more radioactivity is present in the blood of PE$^{32}$P-injected animals that in that of $^{32}$P-treated ones, the difference being highly significant ($p < 0.001$).

Liver. – The data obtained in the liver are shown in Fig. 6.

The difference in slopes is highly significant (Table 7) indicating that the radioactivity disappears much slower from the liver of PE$^{32}$P-treated animals. Since the difference in the amount of radioactivity incorporated into the liver following different treatments is highly significant, it would seem likely that a part of subcutaneously administered PE$^{32}$P is rapidly accumulated in the liver.

Spleen. – The radioactivity data obtained in the spleen are presented in Fig. 7.

Since it has not been possible to find a transformation yielding valid lines for the spleen-data, the computed lines are indicated with dotted lines in order to show their invalidity. Although the lines shown in Fig. 7 are invalid, it is apparent from the data that no major difference exists in the amount of radioactivity incorporated into this organ following the administration of PE$^{32}$P and $^{32}$P respectively.
Fig. 6.
Disappearance of radioactivity from the liver of PE\textsuperscript{32P}-treated (filled circles) and from that of \textsuperscript{32}P-treated (open circles) mice. Route of administration: subcutaneous. The equation of the PE\textsuperscript{32P}-line: \( Y = 2.873 - 0.056 x \), and that of the \textsuperscript{32}P-line: \( Y = 3.008 - 0.348 x \). For further particulars consult Fig. 4.

Fig. 7.
Disappearance of radioactivity from the spleen of PE\textsuperscript{32P}-treated (filled circles) and from that of \textsuperscript{32}P-treated (open circles) animals. Route of administration: subcutaneous. No valid lines could be obtained; therefore dotted lines indicate only approximate disappearance rates.
Uterus. The lines obtained in the uterus are presented in Fig. 8.

It has not been possible to measure the radioactivity on the 16th day of experiment, due to the very low activity present. The difference between slopes is not significant (see Table 7), indicating that the radioactivity disappears from the uterus with the same rate following the administration of PE\textsuperscript{32}P or \textsuperscript{32}P. It is also apparent from Fig. 8, that comparable amounts of radioactivity are incorporated into this organ following the administration of the two substances. In view of the extremely small amount of radioactivity recoverable from the uterus following the administration of PE\textsuperscript{32}P it would also seem feasible to conclude that an accumulation of PEP in the target organ cannot play an important role in the mechanism of prolonged action of this substance.

Kidney. The lines obtained on the analysis of radioactivity-data in the kidneys are shown in Fig. 9.

A comparison of the PE\textsuperscript{32}P- and \textsuperscript{32}P-lines reveals that neither the slopes nor the amount of incorporated radioactivity differ significantly. It seems therefore likely that PE\textsuperscript{32}P is not accumulated in the kidneys to any major extent.

Brain. The regression lines shown in Fig. 10 have been calculated only from the data obtained on the 1st, 2nd and 4th days of experiment. On the 8th and 16th days of experiment the concentration of radioactivity in brain decreases; these points have been omitted from Fig. 10.

![Counts/gm vs Days graph for Uterus](image1)

**Fig. 8.** Disappearance of radioactivity from the uterus of PE\textsuperscript{32}P-treated (filled circles) and from that of \textsuperscript{32}P-treated (open circles) animals. Route of administration: subcutaneous. The equation of the PE\textsuperscript{32}P-line: \(Y = 3.030 - 0.368x\), and that of the \textsuperscript{32}P-line: \(Y = 2.781 - 0.274x\). For further particulars consult Fig. 4.

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Fig. 9.
Disappearance of radioactivity from the kidneys of PE^{32}P-treated (filled circles) and from \(^{32}\)P-treated (open circles) animals. Route of administration: subcutaneous. The PE^{32}P-line has the equation: \(Y = 3.121 - 0.364 \times \) and the \(^{32}\)P-line: \(Y = 2.979 - 0.316 \times \).
For further particulars consult Fig. 4.

Fig. 10.
Uptake of radioactivity in the brain of PE^{32}P-treated (filled circles) and in that of \(^{32}\)P-injected (open circles) animals. Route of administration: subcutaneous. The equation of the PE^{32}P-line is \(Y = 0.912 + 0.157 \times \), and that of the \(^{32}\)P-line: \(Y = 1.512 + 0.128 \times \).
For further particulars consult Fig. 4.
Due to the very small amount of radioactivity found in this organ, it was necessary to combine brains from 4 animals for each estimation. Thus every point shown in Fig. 10 is based only on 5 separate estimations, although they represent 20 animals.

The positive slopes are compatible with the view that radioactive phosphate penetrates the brain only very slowly. The difference between the PE32P- and 32P-slopes is not significant (see Table 7), indicating that the radioactivity penetrates the brain with approximately the same velocity following PE32P- or 32P-administration. However, the difference in y-values is significant ($p < 0.01$), showing that more radioactivity can enter this organ following 32P-injection than after the administration of PE32P. Thus it would appear from the data of Fig. 10 that PE32P penetrates the brain only to a small extent, or possibly not at all.

Residual fraction. – The lines obtained on the residual fraction (which includes all the organs not analyzed before, i.e. carcass, intestines, lungs, etc.) are shown in Fig. 11.

An unsuccessful attempt was made on the residual fractions obtained on the first day to develop anotherashing technique. These values are not comparable with those obtained on the other days of experiment. They have not been incorporated into Fig. 11.

The difference in slope is significant (see Table 7) indicating that the radio-

![Graph](https://example.com/graph.png)

**Fig. 11.**
Disappearance of radioactivity from the residual fraction of PE32P-treated (filled circles) and from that of 32P-treated spayed mice. Route of administration: subcutaneous. The PE32P-line has the equation: $Y = 2.276 - 0.035 \times$, and the 32P-line: $Y = 2.346 - 0.089 \times$. For further particulars consult Fig. 4.
activity disappears – in fact – more slowly from PE32P-treated animals than from 32P-injected ones.

Comments on the data obtained following the subcutaneous administration of PE32P. – Since even on the 16th day of experiment all the animals exhibited an oestrus smear, it is justifiable to conclude that the dose of PEP administered was sufficient to keep the animals in continuous oestrus in the course of the whole experiment. Additional evidence in favour of this conclusion was obtained at the examination of the uteri of the animals, which on the 16th day of experiment still showed signs of maximal stimulation.

A perusal of the data presented in this chapter reveals that in certain organs such as the kidney and the uterus no difference can be demonstrated in the amount of radioactivity incorporated or in the rate of disappearance of radioactivity following the administration of PE32P and of 32P respectively. On the other hand, organs such as the skin, blood, liver and the so-called residual fraction contained more radioactivity following PE32P- than after 32P-injection. In two of these organs, i.e. in the liver and in the residual fraction, even the rate of disappearance of radioactivity was found to be significantly lower in the PE32P-treated group.

These findings point to the fact that more radioactivity is accumulated in the organism of the animals following PE32P- than after 32P-administration. However, the magnitude of this retention cannot be assessed adequately without a closer consideration of the absolute amount of radioactive phosphorus present in the different organs. Therefore in Table 8, the absolute amount of radioactivity found in different organs on the 2nd day of experiment is presented as a percentage of the administered dose.

It would appear from Table 8 that 48 hours following a single subcutaneous injection approximately 60 per cent of the radioactivity administered in form of PE32P can be accounted for. In contrast to this, only approximately 40 per cent of the radioactivity could be recovered from the animals treated with 32P. Apparently, the difference, which amounts to 20 per cent, depends on the accumulation of radioactivity in the blood, liver, skin and residual fraction of PE32P-treated animals. On the hypothesis, that the prolonged biological action of PEP depends on a "depot"-formation somewhere in the organism, each of these organs could be suspected to be of significance for the prolonged duration of effectiveness.

However, the presence in an organ of a high amount of radioactivity following the administration of PE32P cannot be taken as clear-cut evidence indicating the presence of high amounts of PE32P. A considerable part of the radioactivity measured may equally have arisen from radioactive phosphate liberated from PE32P and incorporated into other fractions. Thus before attempting to evaluate the rôle played by these organs as depots for PEP, it seemed to us necessary to obtain some more information on the chemical nature

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Radioactivity recovered from various organs of spayed mice 48 hours after a single subcutaneous injection of labelled poly-oestradiol phosphate (PE$^{32}$P) and of labelled inorganic phosphate ($^{32}$P) respectively.

Data are expressed as percentage of the administered dose.

Total blood volume was assumed to be $1/10$ of the body weight.

<table>
<thead>
<tr>
<th>Organ</th>
<th>PE$^{32}$P per cent recovered</th>
<th>$^{32}$P per cent recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>6.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Liver</td>
<td>6.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Uterus</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Brain</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Skin</td>
<td>10.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Residual fraction</td>
<td>36.7</td>
<td>35.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>61.1</strong></td>
<td><strong>40.8</strong></td>
</tr>
</tbody>
</table>

of radioactivity present in various organs following the administration of PE$^{32}$P.

In our opinion, the data presented in the next section make it highly probable that the radioactivity measured in organs of PE$^{32}$P-treated animals reflects rather closely the amount of PEP present.

2. Evidence suggesting that the radioactivity measured arises mainly from PE$^{32}$P. - Generally speaking, approximately one-third of the total phosphorus present in an organ can be expected to be in form of »acid soluble« phosphorus compounds, whereas another third is made up by »lipid soluble« phosphorus. The last third consists of the so-called »residual phosphorus« which is made up mainly by phosphorus present in nucleic acids and phosphoproteins. This last group is practically insoluble in ice cold trichloroacetic acid, or ethanol-ether. Thus an extraction procedure consisting of extraction by cold trichloroacetic acid (hereafter: TCA) followed by extraction with ethanol-ether will presumably remove the bulk of acid soluble and lipid soluble phosphorus compounds, without removing any appreciable amount of the residual phosphorus. Assuming now, that also PEP were insoluble or at least very sparingly soluble in the solvents mentioned, it would seem possible to remove approximately $2/3$ of the original phosphorus content of an organ without any considerable loss to the PEP-concentration. In this way, it would seem feasible to develop a more specific method for the estimation of PE$^{32}$P in different organs. In order to
test this assumption, *in vitro* as well as *in vivo* recovery experiments on PE$^{32}$P were carried out as follows:

a) **Recovery of PE$^{32}$P added to minced liver tissue.** – In order to explore the utility of the extraction technique, 7200 counts/min. of PE$^{32}$P were added to 20 mouse-livers, homogenized and divided into 40 equal portions. Thus each sample was expected to contain approximately 360 counts/min. Half of the samples were submitted to dry ashing, plated and counted. The analysis of these 20 samples gave 258.4 ± 25.8 (Mean ± S.D.) counts/min. The other half of the samples were extracted by $2 \times 5$ ml. ice cold TCA-solution and by $2 \times 5$ ml. ethanol-ether (1 : 3). The TCA- and ethanol-ether-extracts were discarded and the acid insoluble, lipid insoluble residue was submitted to dry ashing, plated and counted. The analysis of these 20 specimens gave 229.8 ± 30.3 counts/min. It follows from the data presented in Table 1, that under our experimental conditions, the dry ashing technique involves a loss in radioactivity of 27 per cent. When the present data are corrected for this loss, 354.0 counts/min. are obtained in the first group instead of the calculated 360, whereas the TCA-ethanol-ether-extracted group yields 314.8 counts/min. This experiment indicates therefore that the use of TCA-ethanol-ether extraction procedure will only remove 11 per cent of PE$^{32}$P added to minced liver tissue. Thus it follows from the *in vitro* recovery experiment that it is possible to remove the bulk of acid soluble, lipid soluble phosphorus compounds from an organ such as the liver, without losing more than approximately 10 per cent of PE$^{32}$P present. In view of this favourable result it seemed to be of interest to carry out some *in vivo* experiments as follows:

b) **Recovery of PE$^{32}$P from the blood and liver of PE$^{32}$P-treated mice.** – In order to assess the approximate amount of PE$^{32}$P present in the blood and liver of PE$^{32}$P-treated animals, 80 spayed mice were injected subcutaneously with 169 µg./20 gm. body weight of PE$^{32}$P corresponding to 8300 ± 596 counts/min. (mean ± S.D. of 15 estimates). 80 control animals were injected with the same amount of non-labelled PEP, 7900 ± 470 counts/min. (15 estimates) in form of $^{32}$P. The difference between these amounts is not significant ($p > 0.05$). Groups of 20 animals were sacrificed on the 1st, 2nd, 4th and 8th days of experiment. From each animal 0.2 ml. blood was withdrawn, the blood pipetted into 5.0 ml. 10 per cent TCA-solution and the radioactivity remaining in the blood following repeated extractions by TCA and by ethanol-ether, respectively, estimated. The liver was worked up in the same manner as indicated in the *in vitro* recovery experiment.

The concentration of radioactivity in the blood following extraction with TCA and ethanol-ether, is presented in Fig. 12.

It would appear from Fig. 12 that the radioactivity disappears more rapidly from the blood of PE$^{32}$P-treated animals than from that of the controls. The difference between the two slopes is significant (Table 7) and that between the
Disappearance of radioactivity from the acid insoluble-, lipid insoluble fraction of the blood following a single subcutaneous injection of PE$^{32}$P (filled triangles), or that of $^{32}$P (open triangles). The equation of the PE$^{32}$P-line is $Y = 3.192 - 0.568x$, and that of the $^{32}$P-line: $Y = 1.819 - 0.276x$. The PE$^{32}$P-group was injected with 8300 counts/min. per 20 gm. body weight, and the $^{32}$P-group with 7900 counts/min. per 20 gm. body weight. For further particulars consult Fig. 4.

A comparison of »extracted« (Fig. 12) and unextracted (Fig. 5) blood levels of radioactivity in PE$^{32}$P-treated animals reveals no significant difference in slope. (See Table 7). It is also apparent that the concentration of radioactivity in the blood of PE$^{32}$P-treated animals is not reduced appreciably following TCA- and ethanol-ether extraction, whereas the same extraction procedure will remove more than 50 per cent of radioactivity from the blood of $^{32}$P-treated animals. Therefore it would seem justifiable to conclude that the major part of circulating radioactivity in PE$^{32}$P-treated animals – in fact – consists of PE$^{32}$P.

It is obvious, however, that the upper line shown in Fig. 12 does not represent exclusively PE$^{32}$P, but also some other acid insoluble-, lipid insoluble phosphorus compounds, which also may contain a certain amount of radioactivity. Therefore it is not possible to estimate from the data presented in Fig. 12 the »true« concentration of PE$^{32}$P in the blood of the animals. It is possible, however, to calculate certain limits, within which the »true« blood-level of PE$^{32}$P is to be found, if the following assumptions are made:
Assuming that the contribution to the total radioactivity of the upper line in Fig. 12 by the phosphorus incorporated into nucleic acids or into other acid insoluble-, lipid insoluble compounds is very little or nil, it could be reasoned that in the most favourable case all the radioactivity measured will arise from PE\textsuperscript{32}P. Therefore this line confidently can be regarded as the upper limit of the »true« blood concentration of PE\textsuperscript{32}P (PE\textsubscript{max}).

On the other hand, a relatively great contribution by the radioactivity incorporated into the phosphorus of nucleic acids and phosphoproteins must also be taken into consideration. It can be expected that this contribution will be maximal, when all the PE\textsuperscript{32}P will be hydrolyzed into inorganic phosphate instantaneously following subcutaneous administration. In such a hypothetical case experimental conditions in the PE\textsuperscript{32}P- and \textsuperscript{32}P-treated groups will be identical, and the amount of radioactivity incorporated into the acid insoluble-, lipid insoluble fraction of the blood of PE\textsuperscript{32}P-treated animals, must equal that found in this fraction following the administration of \textsuperscript{32}P. Obviously, in practice the radioactivity present in the nucleic acid- and phosphoprotein fractions in the blood of PE\textsuperscript{32}P-treated animals can never reach that found in the acid insoluble-, lipid insoluble fraction in \textsuperscript{32}P-treated animals. Nevertheless, making a generous allowance for the radioactivity present in other form than PE\textsuperscript{32}P in this acid insoluble-, lipid insoluble fraction, by subtracting in Fig. 12 the values of the \textsuperscript{32}P-line from those of the PE\textsuperscript{32}P-line, a new line can be obtained, which will represent the lower limit, (PEP\textsubscript{min}). Obviously, the actual amount of PE\textsuperscript{32}P present in the blood always must exceed the values represented by this hypothetical line. Apparently, the »true« blood level of PE\textsuperscript{32}P must lie somewhere within the area delineated by PEP\textsubscript{max} and PEP\textsubscript{min} as shown in Fig. 13.

![Graph](https://example.com/graph.png)

**Fig. 13.** Area calculated from the data of Fig. 12, within which the »true« disappearance rate of PE\textsuperscript{32}P from the blood is expected to lie. For particulars consult text.
From a comparison of Figs. 5 and 13, it would appear justifiable to assume that the radioactivity measured in the blood of PE³²P-treated animals will reflect rather closely the »true« concentration of circulating PEP.

The data obtained in the acid insoluble-, lipid insoluble fraction of the liver are shown in Fig. 14. Unfortunately, it was not possible to obtain estimates in the ³²P-group on the 8th day of experiment.

The difference in slopes shown in Fig. 14 is highly significant (Table 7). From a comparison of the data presented in Figs. 6 and 14 it also appears that the removal of acid soluble- and lipid soluble phosphorus compounds will not cause any significant change in the disappearance rate of radioactivity from the liver of PE³²P-treated animals (see Table 7). This is circumstantial evidence indicating that measurements of total radioactivity in the liver of PE³²P-treated animals will provide a rather close approximation to the actual amount of PEP present in this organ.

A comparison of Figs. 6 and 14 reveals, that whereas extraction by TCA and ethanol-ether will not influence appreciably the concentration of radioactivity in the liver of PE³²P-treated animals, the same extraction procedure will lead to a great loss in the amount of radioactivity present in the liver of ³²P-injected mice. Thus the difference in the amount of radioactivity incorporated into the liver of PE³²P- and ³²P-treated animals respectively, can be greatly exaggerated by the removal of acid soluble- and lipid soluble phosphorus compounds. This finding favours the view that the radioactivity measured in the liver of PE³²P-treated animals consists essentially of PE³²P.

Thus from the evidence presented in this section it would appear justifiable to conclude that measurement of radioactivity in various organs of PE³²P-treated animals will reflect – within certain limits – the approximate amount of PEP incorporated into the organ.

Fig. 14. Disappearance of radioactivity from the acid insoluble-, lipid insoluble fraction of the liver following a single subcutaneous injection of PE³²P (filled triangles) and of ³²P (open triangles) respectively. The equation of the PE³²P-line is: \( Y = 2.888 - 0.096 \times \), and that of the ³²P-line: \( Y = 2.753 - 0.317 \times \). For further particulars consult Figs. 4 & 12.
3. Distribution of radioactivity following intravenous administration of PE\textsuperscript{32}P. - Examination of the data obtained on the first day following subcutaneous administration of PE\textsuperscript{32}P (page 20) reveals that at this time already 90 per cent of the administered radioactivity disappeared from the site of injection. This fact would seem to indicate that the skin-depot at the site of injection has only a limited significance for the prolonged action of PEP. This conclusion finds further support in previously reported data (Diczfalusy, 1954) indicating that the intravenous administration of PEP also gives rise to a prolonged duration of effectiveness. Thus, in order to find out, whether the intravenous administration of PEP is also followed by the formation of depots in the organism, it seemed desirable to study the distribution of radioactivity in spayed mice following the intravenous administration of PE\textsuperscript{32}P.

In this part of our study, the same number of animals and the same design were used as in the subcutaneous experiment. Radioactivity was administered in a volume of 0.1 to 0.2 ml. (depending on body weight) into the tail vein. This experiment as well as that described in section 2 (page 28) was started later than the subcutaneous experiment; therefore in order to administer approximately the same amount of radioactivity it was necessary in both experiments to administer as much as 169 µg. PE\textsuperscript{32}P per 20 gm. body weight, corresponding to 8300 ± 596 counts/min. (the S.D. is based on 15 estimates). The control group was injected with the same amount of non-labelled PEP + 7900 ± 470 counts/min. (S.D. based on 15 estimates) in form of inorganic phosphate (\textsuperscript{32}P). The difference between these amounts of radioactivity is not significant (p > 0.05). It seems therefore justifiable to conclude that the PE\textsuperscript{32}P- and \textsuperscript{32}P-groups were injected with the same amount of radioactivity also in the intravenous experiment. A further comparison of the amounts of \textsuperscript{32}P administered subcutaneously and intravenously respectively, reveals no significant difference (p > 0.05). However, the difference between the amounts of PE\textsuperscript{32}P administered subcutaneously and intravenously, respectively, is significant (p < 0.05), although just barely significant. Nevertheless, these two experiments are not strictly comparable. Since the intravenously treated animals obtained approximately 8 per cent more radioactivity than those treated subcutaneously, this fact should be borne in mind whenever these two groups will be compared.

Similarly to the findings obtained in the subcutaneous experiment, all animals exhibited a positive vaginal smear and a hypertrophied uterus even on the 16th day following a single intravenous injection of PE\textsuperscript{32}P. This confirms previous findings, indicating that intravenously administered PEP is capable of eliciting a prolonged oestrogenic effect. However, due to the much higher dosage of PE\textsuperscript{32}P employed in the intravenous experiment, the present data do not permit a direct comparison of the biological effectiveness of intravenously and subcutaneously administered PEP.
In the intravenous experiment, radioactivity measurements were limited to the analysis of the following organs:

- blood
- liver
- spleen
- residual fraction.

It should also be pointed out, that the »residual fraction« of the intravenous experiment contains organs, such as kidney, uterus, etc. which have been analyzed separately in the subcutaneous experiment. It would appear, however, from the data presented in Table 8, that the contribution by these organs to the total radioactivity found in the whole organism is negligible.

**Blood.** – The data obtained on the analysis of the blood are presented in Fig. 15.

The dotted line for $^{32}$P indicates that this regression line is not valid. This line should be considered therefore only as an approximate indication of the disappearance of intravenously administered $^{32}$P from the blood. Thus this invalid regression line cannot be compared with the line obtained in PE$^{32}$P-treated animals. Nevertheless, the difference of the individual points on the $^{32}$P- and PE$^{32}$P-lines respectively, can be checked in the usual $t$-test. The difference between PE$^{32}$P- and $^{32}$P-blood levels on the 1st to 4th days is significant at the 99.9 per cent probability level ($p < 0.001$), whereas that on the 8th day is significant at the 95 per cent level ($p < 0.05$), indicating that the blood of PE$^{32}$P-treated animals does – in fact – contain more radioactivity than that of $^{32}$P-injected animals, also when the intravenous route of administration is

![Graph](image)

**Fig. 15.**

Disappearance of radioactivity from the blood of PE$^{32}$P-treated (filled circles), and from that of $^{32}$P-injected (open circles) animals. Route of administration: intravenous. The PE$^{32}$P-line has the equation: $Y = 2.908 - 0.457 x$. Dotted line indicates invalid regression line. For the amount of radioactivity administered consult Fig. 12. For further particulars consult Fig. 4.
employed. Thus from the data presented in Fig. 15 it would appear that a relatively high amount of PE$^{32}$P is present in the circulation during a relatively long time, even after intravenous administration.

The difference between the slopes of the lines obtained in the blood of PE$^{32}$P-treated animals following subcutaneous and intravenous administration respectively is not significant (Table 7). This seems to indicate that the disappearance rate of PE$^{32}$P from the blood is the same following subcutaneous and intravenous administration.

A comparison of the corresponding $\bar{y}$-values reveals, however, a significant difference ($p < 0.001$) indicating that the blood level of PE$^{32}$P in intravenously treated animals is significantly lower than in subcutaneously treated animals, in spite of the fact, that the former group received approximately 8 per cent more radioactivity.

In contradistinction to the behaviour of PE$^{32}$P administered by different routes, the blood of $^{32}$P-treated mice contains more radioactivity following intravenous- than after subcutaneous administration. Using the usual $t$-test, a highly significant difference can be calculated ($p < 0.001$) for the data obtained on each day of the experiment. (Compare Figs. 5 and 15). Since in the subcutaneous experiment less than 1 per cent of the administered radioactivity could be recovered from the site of injection 24 hours following a single subcutaneous injection, the significantly lower blood concentration of $^{32}$P found in the subcutaneous experiment, cannot be explained by assuming that less radioactivity reached the circulation following subcutaneous than after intravenous injection. Since, furthermore, the difference in administered $^{32}$P-doses ($7.848 \pm 306$ and $7.900 \pm 470$ counts respectively) is not significant, it seems justifiable to conclude that the disposal of $^{32}$P administered as orthophosphate changes with the route of administration. However, under our experimental conditions $^{32}$P was always administered together with non-labelled PEP. Thus the validity of the above conclusion is subject to the assumption that the presence of PEP has no influence whatsoever on the disposal of $^{32}$P.

Liver. – The results of the analyses on the liver are shown in Fig. 16.

Similarly to the blood data, no valid line could be obtained in the liver of $^{32}$P-treated animals. It is apparent from the data shown in Fig. 16 that much more radioactivity is present in the liver of PE$^{32}$P- than in that of $^{32}$P-treated animals. The difference between the data obtained following different treatments is highly significant ($p < 0.001$) on each day of the experiment.

The regression coefficient for the data of PE$^{32}$P-treated animals shown in Fig. 16 is higher than that obtained in the liver of PE$^{32}$P-treated animals following subcutaneous injection (see Table 7). The difference is significant on the 95 per cent probability level ($p < 0.05$). Thus it would appear probable that intravenously administered PE$^{32}$P disappears from the liver more rapidly than the subcutaneously administered substance.
Disappearance of radioactivity from the liver of PE$^{32}$P-treated (filled circles) and from that of $^{32}$P-treated (open circles) animals. Route of administration: intravenous. The equation of the PE$^{32}$P-line is: $Y = 3.710 - 0.103x$. The dotted line indicates invalidity. For the amount of radioactivity administered consult Fig. 12, for further particulars see Fig. 4.

Intravenously administered PE$^{32}$P accumulates in the liver to a very high extent. Whereas only 8 per cent of the administered radioactivity can be recovered from the liver following subcutaneous injection of PE$^{32}$P, corresponding figure following intravenous administration amounts to approximately 40 per cent.

Finally, a comparison of the amount of radioactivity taken up by the liver following subcutaneous and intravenous administration of $^{32}$P (Figs. 6 and 16) reveals, that in general the liver contains more radioactivity following intravenous than after subcutaneous injection. The difference is significant on the first to eighth days ($p < 0.01$), but not on the 16th day of experiment.

Spleen. – The results obtained in the spleen following intravenous administration of radioactivity are shown in Fig. 17.

It appears from Fig. 17 that more radioactivity is present in the spleen following PE$^{32}$P- than after $^{32}$P-administration: the difference between the individual points being significant at least at the 99 per cent probability level. The regression coefficient for the PE$^{32}$P-line has been estimated as $b = 0.005 \pm 0.017$. Apparently, intravenously administered PE$^{32}$P is stored in the spleen similarly to the situation found in the liver.
Fig. 17.
The fate of radioactivity in the spleen of PE\textsuperscript{32}P-treated (filled circles) and in that of \textsuperscript{32}P-injected (open circles) animals. Route of administration: intravenous. The equation of the PE\textsuperscript{32}P-line is: \( Y = 3.233 + 0.005 \times \). For the amount of radioactivity administered consult Fig. 12; for further particulars Fig. 4.

Fig. 18.
Disappearance of radioactivity from the residual fraction of PE\textsuperscript{32}P-treated (filled circles) and from that of \textsuperscript{32}P-treated (open circles) animals. Route of administration: intravenous. The equation of the PE\textsuperscript{32}P-line: \( Y = 2.332 - 0.024 \times \), and that of the \textsuperscript{32}P-treated line: \( Y = 2.474 - 0.052 \times \). For the amount of radioactivity administered consult Fig. 12; for further particulars Fig. 4.

It is also obvious from a comparison of Figs. 7 and 17, that significantly more radioactivity is demonstrable in the spleen following intravenous than after subcutaneous administration of PE\textsuperscript{32}P. In this connection it must be borne in
mind, however, that although the relative concentration of PE$^{32}$P per gm. spleen is very high following intravenous administration, the absolute amount of radioactivity incorporated by this relatively small organ is – in fact – very limited indeed, since it does not exceed 2 per cent of the total administered dose.

Residual fraction. – The lines calculated from the data of the residual fraction are presented in Fig. 18.

Neither the difference in slopes nor that in the amount of radioactivity is significant (Table 7).

A comparison of the slope obtained in the intravenously treated animals with that found in the subcutaneous experiment, does not indicate any significant difference, neither in PE$^{32}$P-, nor in $^{32}$P-treated animals.

4. Comparison of the total amount of radioactivity retained in the organism following subcutaneous and intravenous administration of PE$^{32}$P and $^{32}$P respectively.

When the total amount of radioactivity, which can be recovered from the whole organism following the administration of PE$^{32}$P and $^{32}$P by different routes is considered, some interesting observations can be made. Fig. 19 indicates the total amount of radioactivity (expressed as a percentage of the administered dose) which could be found in the whole body of the animals on different days of the experiment. Since the data on the residual fractions from the first days of the subcutaneous experiment are missing, the lines are based only on the data obtained on the 2nd to 16th days.

Fig. 19 A shows a comparison of the amount of radioactive phosphorus re-

![Figure 19](image-url)
coverable from the animals injected with the same amount of $^{32}$P intravenously (upper line) and subcutaneously (lower line) respectively. The difference is striking, whereas 40 per cent of the administered $^{32}$P-dose can be recovered on the 2nd day, and 20 per cent on the 16th day following subcutaneous administration, corresponding percentages are 56 on the second, and 40 per cent on the 16th day following intravenous administration of the same amount of radioactivity. The reason for this great discrepancy is not well understood. A more detailed study of the mechanism by which intravenously administered $^{32}$P is accumulated in the organism would seem to be of particular interest; this problem was, however, beyond the scope of the present investigation.

Fig. 19 B shows the total amount of radioactivity present in the body of PE$^{32}$P-treated animals. Apparently, much more radioactivity is retained in the organism following the administration of PE$^{32}$P than after the injection of $^{32}$P. Since, however, approximately 60 per cent radioactivity can be recovered on the 2nd, and 32 per cent on the 16th day following a single subcutaneous injection of PE$^{32}$P, it is evident that a considerable portion of the administered radioactivity has been excreted by these animals. Since it is unlikely that unchanged PEP will be excreted in the urine, it would seem justifiable to conclude that PE$^{32}$P is slowly hydrolyzed in the organism of the mouse. It is of interest to note, that following intravenous administration of PE$^{32}$P much more radioactivity is retained in the organism than after a subcutaneous injection; as much as 90 per cent of the administered dose seems to be recoverable on the 2nd day of experiment, and approximately 60 per cent is still present on the 16th day.

Fig. 19 C shows two constructed lines. These dotted lines indicate the total amount of radioactivity found in the whole organism, following the subcutaneous administration of $^{32}$P (lower line) and of PE$^{32}$P (upper line), respectively. However, the amount of radioactivity originally present in the liver of these animals has been replaced by that found in the liver of intravenously treated animals. In other words, these lines represent radioactivity data from subcutaneously injected »animals« submitted to hepatectomy and subsequently implanted with the liver of an intravenously injected animal.

The line obtained in this way from the data of the $^{32}$P-experiments agrees closely with the original subcutaneous $^{32}$P-line shown in Fig. 19 A. On the other hand, the line constructed from the data of PE$^{32}$P-treated animals approaches rather closely the original intravenous PE$^{32}$P-line shown in Fig. 19 B. It seems likely therefore, that the difference in the total amount of radioactivity retained in the organism following subcutaneous or intravenous administration of PE$^{32}$P depends mainly — although probably not entirely — on the higher concentration of PE$^{32}$P in the liver of intravenously treated animals. Obviously, a similar mechanism cannot offer an acceptable explanation for the greater retention of intravenously administered $^{32}$P. It is therefore apparent, that dif-
different mechanisms are involved in the accumulation of intravenously administered radioactivity, when $^{32}$P or when PE$^{32}$P is injected.

5. Rôle of the reticuloendothelial system in the uptake of PE$^{32}$P by the liver. - The concentration of PEP in the blood must be very high shortly after its intravenous administration. However, 24 hours following a single intravenous injection of PE$^{32}$P, significantly less radioactivity is found in the blood than after the subcutaneous administration of the same amount of PE$^{32}$P. The reason for the fact, that at this time the major part of intravenously administered PE$^{32}$P has already been removed from the circulation, must be sought in the relatively great accumulation of this substance in the liver. The rapid uptake of PE$^{32}$P in the liver would seem to point to the possibility that the concentration of circulating PEP is regulated by the action of the liver. This regulatory action of the liver may depend either on the function of the reticuloendothelial cell elements of the liver, or/and on that of the liver cells. The simultaneous accumulation of intravenously administered PE$^{32}$P in the spleen seems to favour the first hypothesis. If this were true, it could be expected that a blockade of the reticuloendothelial system (hereafter RES) would partially inhibit the uptake of circulating PE$^{32}$P by organs rich in reticuloendothelial cell elements. Such an inhibition must then result in a higher blood concentration of PE$^{32}$P in »blocked« animals.

Evidence presented below seems to be consistent with this hypothesis.

Design of experiment: 60 spayed mice have been divided into 3 equal groups. Each animal was injected intravenously with 4000 counts/min. per 20 gm. body weight PE$^{32}$P. 20 animals served as a control group (group a). The animals in the second group (group b) were pretreated 2 hours before the PE$^{32}$P injection with a single intravenous dose of 0.2 ml. of india ink (diluted with an equal volume of saline and filtered). The third group (group c) was pretreated intravenously with 6.0 mg. per 20 gm. body weight non-labelled PEP, also 2 hours prior to the administration of PE$^{32}$P. One hour following the administration of radioactivity the animals were sacrificed. 0.2 ml. blood was withdrawn, plated, dried and counted. The liver and spleen were rapidly dissected out, weighed and prepared for radioactivity measurements, following dry ashing.

The results of the »blockade«-experiment are compiled in Fig. 20, where radioactivity is expressed as counts/gm. organ and counts/ml. blood, respectively.

It appears from Fig. 20, that much more radioactivity is taken up in the liver of control animals, than in that of »blocked« ones; the difference between groups a and b, as well as that between groups a and c is highly significant ($p < 0.001$).

Thus it would appear from these data that PEP is capable of blocking the uptake of PE$^{32}$P by the liver in the same way as india ink does. Since the latter is known to be preferentially taken up by the RES, it seems reasonable to assume
that the RES is involved in some way in the high uptake of PEP by the liver.

It may be expected, that a blockade of the liver will result in a higher blood level of radioactivity. Indeed the blood concentration of PE\textsuperscript{32}P is much higher in the groups pretreated with india ink or with non-labelled PEP, than in the control group; again the difference between groups \(a\) and \(b\), as well as that between \(a\) and \(c\) is highly significant (\(p < 0.001\)).

It appears also from Fig. 20 that no blockade effect similar to that found in the liver can be demonstrated in the spleen. This organ is also known to be rich in RES; nevertheless the concentration of PE\textsuperscript{32}P in the spleen is higher rather than lower following the administration of india ink or of PEP. In our opinion, this apparent accumulation of radioactivity in the spleen following blockade can be explained by the presence of a considerable amount of blood in this organ, which exhibits a high radioactivity 1 hour following the administration of PE\textsuperscript{32}P. It is therefore feasible to assume that there may be a certain degree of RES-blockade even in this organ, which, however, cannot

Fig. 20.
Influence of a previous »blockade« of the reticuloendothelial cell system upon the concentration of radioactivity in different organs of spayed mice, injected intravenously with labelled poly-oestradiol phosphate (PE\textsuperscript{32}P).

Group \(a\) (open columns): Control groups sacrificed 60 minutes following the administration of 4000 counts/min. of PE\textsuperscript{32}P.

Group \(b\) (filled columns): Same as \(a\), but pretreated with an intravenous dose of 0.2 ml. of india ink 2 hours prior to the administration of PE\textsuperscript{32}P.

Group \(c\) (dotted columns): Same as \(a\), but pretreated with an intravenous dose of 6 mg. of non-labelled PEP 2 hours prior to the administration of PE\textsuperscript{32}P.

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In our opinion, this apparent accumulation of radioactivity in the spleen following blockade can be explained by the presence of a considerable amount of blood in this organ, which exhibits a high radioactivity 1 hour following the administration of PE\textsuperscript{32}P. It is therefore feasible to assume that there may be a certain degree of RES-blockade even in this organ, which, however, cannot
be demonstrated at this early stage due to the overlapping high radioactivity concentration of the contaminating blood.

Thus in view of the evidence presented in Fig. 20, it would seem plausible to assume that the liver is an organ of relatively great importance in the regulation of the circulating amount of PEP. Whether this regulatory function is exerted only by the RES in the liver, or by the liver cells, or possibly by both of them, remains to be established. Data obtained by Dr. E. Odeblad on contact autoradiographic examination of livers from PEP32P-treated mice indicate that radioactivity is present in the reticuloendothelial cells and probably also in the liver cells. Thus the evidence presented in this section, and the data obtained by Dr. Odeblad make it highly probable that the reticuloendothelial system is involved in the mechanism by which PEP is taken up in the liver.

6. Possible rôle of the liver in the prolonged duration of effectiveness of PEP. -

The preferential uptake by the liver of intravenously administered PEP raised the question as to the significance of the PEP-depot in the liver for the duration of oestrogenic effect elicited by this substance. Since only approximately 6 per cent of subcutaneously administered PEP could be recovered from the liver, whereas more than 30 per cent of the intravenously administered substance was taken up by this organ, it was hoped that a comparison of the duration of oestrogenic effect of PEP administered by these two routes may provide significant information on the rôle played by the PEP-depot of the liver in the prolongation of oestrogenic effect. In addition to the subcutaneous- and intravenous routes of administration, the effect of intrasplenically injected PEP was also investigated. Intrasplenic injection was carried out following laparotomy. The purpose of the incorporation of the last group was an attempt to obtain the highest possible accumulation of PEP in the liver.

This experiment was designed as a multiple bioassay, as follows:

120 adult female mice were spayed. After the elapse of 14 days the animals were divided into 6 groups with 20 mice each. PEP was administered subcutaneously, intravenously and intrasplenically, by each route at two dose levels, the higher dose being twice the lower. Smears were taken daily and the duration of oestrogenic activity (in days) recorded in each animal. The data are presented in Table 9, where the duration of effectiveness is expressed in log days. The logarithmic transformation was employed since under our experimental conditions straight lines with stable variance could be obtained over a wide range of doses, when log dose was plotted against log days. This is keeping with the view expressed by Gaddum (1958), that for the measurement of duration time, log time is a useful effect metamer.

5. Personal communication.
It appears from the data compiled in Table 9 that a few animals died during the experiment in almost each of the experimental groups.

The analysis of variance for the data of Table 9 is presented in Table 10. From an examination of the ratio of variance due to parallelism and to error, it would appear that the hypothesis of parallelism is not contradicted by the experimental data. Consequently a common regression coefficient was calculated. The subcutaneously administered preparation was considered as »standard«, whereas the intravenously and intrasplenically injected substances as »unknown«. The estimate of relative potency (R) was calculated by using

Table 9.
Duration of oestrogenic effect of poly-oestradiol phosphate (PEP) administered by different routes to spayed mice.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>PEP administered (μg.)</th>
<th>Number of animals</th>
<th>Mean duration of effectiveness ± S. D. (log days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>4.9</td>
<td>18</td>
<td>1.099 ± 0.072</td>
</tr>
<tr>
<td></td>
<td>9.8</td>
<td>17</td>
<td>1.272 ± 0.063</td>
</tr>
<tr>
<td>Intravenous</td>
<td>8.0</td>
<td>16</td>
<td>1.116 ± 0.038</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>17</td>
<td>1.368 ± 0.038</td>
</tr>
<tr>
<td>Intrasplenic</td>
<td>8.0</td>
<td>20</td>
<td>1.067 ± 0.131</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>13</td>
<td>1.286 ± 0.035</td>
</tr>
</tbody>
</table>

Table 10.
Analysis of variance for the data of Table 9.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>1.376</td>
<td>1.376</td>
</tr>
<tr>
<td>Route of administration</td>
<td>2</td>
<td>0.100</td>
<td>0.050</td>
</tr>
<tr>
<td>Parallelism</td>
<td>2</td>
<td>0.032</td>
<td>0.016</td>
</tr>
<tr>
<td>Between doses</td>
<td>5</td>
<td>1.508</td>
<td>0.302</td>
</tr>
<tr>
<td>Error (within doses)</td>
<td>95</td>
<td>0.985</td>
<td>0.010</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>2.043</td>
<td>—</td>
</tr>
</tbody>
</table>
the log ratio of potencies (M) estimated with Gaddum’s (1933) equation as

\[ M = \bar{x}_S - \bar{x}_U - \frac{\bar{y}_S - \bar{y}_U}{b} \]  

where \( \bar{x}_S \) and \( \bar{x}_U \) indicate the mean dose, and \( \bar{y}_S \) and \( \bar{y}_U \) the mean response to standard and unknown preparation respectively, and \( b \) is the common slope calculated from all the data shown in Table 9.

Fiducial limits of error were calculated at a probability of 0.95 according to the formula given by Finney (1952), the limits to \( (M - \bar{x}_S + \bar{x}_U) \) being equal to

\[ \left[ M - \bar{x}_S + \bar{x}_U \pm \frac{ts}{b} \left\{ (1-g) \left( \frac{1}{N_S} + \frac{1}{N_U} \right) + \frac{(M - \bar{x}_S + \bar{x}_U)^2}{\sum S_{xx}} \right\}^{1/2} (1-g) \]  

where \( N_S \) and \( N_U \) indicate the total number of animals on standard and unknown preparations respectively, \( t \) is the 0.95 probability deviate with the number of d.f. for the error mean square (Table 10) and \( s \) is the square root of the error mean square; \( g \) indicates Finney’s \( g \)-criterion for the significance of the regression coefficient, calculated as \( g = s^2 \cdot t^2 / b^2 \cdot S_{xx} \), the symbol \( \pm \) indicates the two alternate operations (addition and subtraction respectively) for the upper and lower fiducial limits of error, and \( \sum S_{xx} \) stands for summation extended over all data.

Using the formulae (4) and (5), the relative potency of the intravenously administered preparation can be estimated to be 73.7 per cent of that of the subcutaneously injected PEP, with fiducial limits of error at 63.5 and 89.7 per cent. A similar calculation for the intrasplenic administered substance gives a relative potency of 59.8 per cent, with fiducial limits at 50.5 and 72.9 per cent. Since the actual potency can be assumed to lie within the fiducial limits 95 times out of 100 it can be concluded that both intravenously and intrasplenically administered PEP exhibits a shorter duration of effectiveness than the subcutaneously administered preparation.

From the relative potencies calculated above it would appear that intrasplenic administration results in a still shorter duration of oestrogenic effect than intravenous injection. From the data of Table 9 the potency of intrasplenic administered PEP can also be calculated in terms of the intravenously injected preparation. Using such a calculation, the potency of intrasplenic administered PEP can be asserted to lie between 66.6 and 95.1 per cent of that of the intravenously administered one, with a relative potency of 81.1 per cent. It is therefore justifiable to conclude that intrasplenic administration of PEP results in a shorter duration of effectiveness than intravenous administration.

The relatively shorter oestrogenic effect of intravenously administered PEP as compared to that of the subcutaneously injected substance indicates that there is no positive correlation between the duration of effectiveness on the
one hand, and the amount of PEP incorporated into the liver, on the other hand. Since intrasplenic injection of PEP exhibited a still shorter duration of effectiveness than the intravenously administered preparation, the possibility was considered that this shorter duration of effectiveness may partly depend on a still greater accumulation of PEP in the liver, when injected intrasplenic.

In order to test this hypothesis, the following experiment was carried out:

60 spayed mice were divided into three groups of 20 animals each and injected subcutaneously, intravenously and intrasplenic respectively with 13.4 µg. PE32P per 20 gm. mouse, corresponding to 4940 counts/min. The animals were sacrificed 24 hours following the administration of PE32P, and the radioactivity present in the liver and spleen of the animals was estimated. Results are shown in Table 11.

The difference between the amount of radioactivity incorporated into the liver following intravenous and intrasplenic administration of PE32P respectively, is significant on the 99.9 per cent probability level (p < 0.001) indicating that less radioactivity is recoverable from the liver following intrasplenic- than after intravenous injection. This finding does not harmonize with the hypothesis that there is a negative correlation between the amount of PEP incorporated into the liver on the one hand, and the duration of effectiveness on the other hand. No theory can be offered at present, which would explain satisfactorily the relationship between the data of Table 9 and 11. For a better understanding of the fate of intrasplenic administered PEP, an analysis of the total amount of PEP found in the organism on different days following this mode of administration would appear to be of paramount importance.

It would also appear from the data of Table 11, that only approximately 4 per cent of the subcutaneously administered PE32P can be recovered from the liver. In contrast to this, following intravenous- and intrasplenic administration, 76 and 47 per cent respectively, of the administered dose can be recovered from

Table 11.
Concentration of radioactivity in the liver and spleen of spayed mice 24 hours following the administration of 4940 counts/min. poly-oestradiol phosphate (PE32P) by different routes.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Number of animals</th>
<th>Counts/min./gm. organ ± S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>19</td>
<td>207.2 ± 64.8</td>
</tr>
<tr>
<td>Intravenous</td>
<td>20</td>
<td>3760 ± 611</td>
</tr>
<tr>
<td>Intrasplenic</td>
<td>19</td>
<td>2317 ± 456</td>
</tr>
</tbody>
</table>

45
the liver. Since in the intravenously and intrasplenically injected animals the duration of effectiveness is definitely shorter than in the subcutaneously injected group (Table 9), the accumulation of PE32P in the liver of the former groups must be interpreted on the assumption that the major part of PEP incorporated into the liver probably cannot be utilized effectively for prolonging the oestrogenic action of the preparation. Such an assumption is also supported by the slow disappearance rate of radioactivity from the liver of intravenously- as well as from that of subcutaneously treated animals.

Thus the balance of evidence seems to indicate that the PEP-depot formed in the liver is passive rather than active, as far as its ability to prolong the duration of oestrogenic effect is concerned.

7. Presumed rôle of the blood in the prolonged duration of effectiveness of PEP.

The bulk of evidence presented in the preceding sections does not speak in favour of the view that a PEP-depot present in the liver, residual fraction, or in the skin would be responsible for the prolonged effect of this substance. Therefore as a working hypothesis it was assumed that the duration of effectiveness may be a function of the amount of circulating PEP. Such an hypothesis may offer an explanation for the shorter duration of oestrogenic effect following intravenous than after subcutaneous administration of PEP. Significantly less PE32P was found in the blood of intravenously treated animals than in that of subcutaneously injected ones. In order to test this hypothesis, it seemed desirable to demonstrate that a) a definite correlation exists between the blood level and the amount of PEP administered, and that b) there is a definite correlation (within a wide range of doses) between the amount of PEP administered and its duration of effectiveness.

a) correlation between administered dose and blood level of PEP. – From 6 different experiments it was possible to calculate the amount of PEP – expressed as a percentage of the administered dose – present in the blood and liver of the animals 24 hours following a single subcutaneous or intravenous injection. The results are presented in Fig. 21.

It would seem from Fig. 21 that – within the range of doses studied – the amount of PE32P present in the blood represents a rather constant proportion of the administered dose following subcutaneous as well as intravenous administration. This is equivalent to the statement that a tenfold increase in administered dose can be expected to give rise to a tenfold increase in PEP-concentration in the blood.

A similar relationship seems also to exist in the liver of subcutaneously-, but not in that of intravenously injected animals. It would seem from the data presented in Fig. 21 that the proportion of PE32P taken up by the liver following intravenous injection bore an inverse relationship to the adminis-
Fig. 21.

Amount of poly-oestradiol phosphate (PEP) – expressed as a percentage of the administered dose – present in the blood (upper figure) and in the liver (lower figure) of spayed mice, 24 hours following the administration of different doses of PEP. Open squares indicate data of subcutaneous experiments, filled squares of intravenous experiments. The dose-scale is logarithmic.

A possible explanation for this fact may probably be sought in a »blockade«-effect exerted by PE32P itself in a manner similar to that presented in Fig. 20.

Thus the experiment shown in Fig. 21 seems to indicate that there is an approximately constant relationship between the blood concentration of PEP on the one hand, and the amount of PEP administered, on the other hand.

b) Correlation between administered dose of PEP and its duration of effectiveness. – Five groups of 18 spayed mice each, were injected subcutaneously with a single dose of 2, 4, 8, 16 and 32 µg. of PEP, and the duration of effectiveness was recorded in the same way as indicated on page 43 for the data of Table 9. The results are presented in Fig. 22, where the mean duration time (in log days) is plotted against log dose of PEP administered.

An analysis of variance for the data of Fig. 22 could not reveal any significant evidence against linearity. It seems therefore warranted to assume that – within the range of doses investigated – the relationship between the logarithm of the duration time and log dose of PEP is linear. Thus our working hypothesis that the blood concentration is related to the duration of effectiveness of PEP does not seem to be contradicted by the experimental data. It must be emphasized, however, that the above working hypothesis is subject to the assumption that the disappearance rate of PEP from the blood is not influenced to any appreciable extent by the amount of PEP administered.
The duration of oestrogenic effect in spayed mice following a single subcutaneous injection of various doses of poly-oestradiol phosphate (PEP). Both scales are logarithmic. The equation of the line is: $Y = 0.707 + 0.468 \times$. Each point represents the mean of 18 mice.

VI. DISCUSSION

During the past twenty years considerable success has been achieved in steroid therapy by the introduction of a variety of hormone preparations with prolonged duration of effectiveness. Such a prolonged effect often can be obtained by the introduction of certain chemical groupings into the steroid molecule. Thus esterification may increase the duration of effectiveness of a steroid very considerably. It is generally agreed that the prolonged duration of effectiveness of such hormone preparations depends essentially on a slow liberation of the biologically active steroid from the ester-bond.

When discussing the prolonged action of PEP, it must be borne in mind, that this substance is also a steroid-ester, or more precisely, a polyester. Its prolonged oestrogenic effect may therefore depend on the continuous, slow liberation of small quantities of oestradiol-17β somewhere in the organism. Although PEP is extremely resistant to acid- or alkali-hydrolysis, and to degradation in vivo (see Section 2 in Chapter 5), clinical experiments to be reported elsewhere (Diczfalusy & Westman, 1956) do indicate, that greatly increased quantities of oestrone, oestradiol-17β and of oestriol can be isolated from the urine of ovariectomized women during a long period of time (45 to 125 days) following the subcutaneous administration of a single dose of PEP.

Whereas there is general agreement on the fact that a slow hydrolysis of steroid esters in the organism is – in fact – a prerequisite for a prolonged action
of this type, considerable uncertainty seems to exist as to the tissue concerned with this hydrolysis.

With regard to PEP it could be suggested, that the major site of its hydrolysis is a) the site of injection, b) in target organs, such as the uterus, c) in the liver, d) in organs constituting the so-called residual fraction and e) in the blood.

a) The fact that intravenously administered PEP is also effective in eliciting a prolonged duration of effectiveness, speaks against the subcutaneous depot as a place of major importance for this process. In addition, evidence has been presented here that 90 per cent of the administered PEP-dose disappears from the subcutaneous depot within 24 hours.

b) It is also clear from the data presented in this study that only negligible amounts of PEP\(^{32}\) (less than 0.003 per cent of the administered dose) are recoverable from the uterus. Furthermore, the disappearance rate of this small amount does not differ from that of \(^{32}\)P in the same organ, or from that of PE\(^{32}\)P in some other organs, such as the kidney. Thus the hypothesis, that the prolonged action of PEP is due to the accumulation and subsequent degradation of this substance in the uterus, appears to be very unlikely.

c) It follows from the disappearance rate of PEP from the liver (Figs. 6, 14 and 16) that in this organ the liberation of oestradiol-17\(\beta\) must be very limited indeed. It is also apparent from the data that considerable amounts of PEP must be present in the liver at a time when oestrus smears are no longer demonstrable. Consequently, only subtreshold amounts of oestradiol can be liberated from the liver-depot during this period.

d) In view of the disappearance rate of PEP from the so-called residual fraction, it might be suggested, tentatively, that in the organs contributing to this fraction, conditions are essentially similar to those found in the liver, as far as the hydrolysis of PEP is concerned. It must be realized, however, that the validity of such a statement is greatly limited by the heterogeneity of this residual-fraction. Apparently, with the technique employed in this work, it is not possible to demonstrate a rapid disappearance of PEP from a relatively small organ making up only a minor part of this fraction.

e) Last, but not least, the fact that a high level of PEP, accompanied by a relatively rapid disappearance rate was only found in the blood, would seem to favour the view, that the main site of liberation of oestradiol from PEP is presumably in the blood.

It is therefore suggested, tentatively, that the amount of circulating PEP may be instrumental – through its continuous, slow degradation into oestradiol – in maintaining a prolonged vaginal oestrus in PEP-treated mice.
The distribution of administered poly-oestradiol phosphate (PEP) in spayed mice was studied, using PEP tagged with radioactive phosphorus (PE\textsuperscript{32}P). On different days following the administration of PE\textsuperscript{32}P, the concentration of radioactivity in various organs was compared with that found in organs of control animals injected with the same amount of radioactivity, administered as orthophosphate (\textsuperscript{32}P). The results were as follows:

1. Some of the variables influencing the estimation of radioactivity under our experimental conditions, such as background, variation in efficiency of the tube and absorption losses in sample, have been examined. Corrections have been introduced in order to compensate for the losses encountered in the procedure.

2. The radioactivity data have been adjusted for differences in organ weight by assuming proportionality. All radioactivity data are expressed as counts/minute per gm. organ weight. This decision was motivated by the finding that the gain in precision due to adjustment by proportionality was found to be comparable with that obtained following adjustment by covariance analysis. The use of unadjusted data (counts/minute per organ) resulted in a loss in precision.

3. Attempts have been made to straighten the radioactivity curves obtained by plotting the radioactivity in different organs against the days of experiment. It was found that when log counts/unit weight of organ were plotted against the square root of \( t + 0.75 \) (\( t \) being the time-interval between injection of PEP and sacrifice of animals, measured in days), in the great majority of the organs examined, statistically valid straight lines could be obtained.

4. Following subcutaneous injection, the bulk of radioactivity administered as PE\textsuperscript{32}P or \textsuperscript{32}P disappears rather rapidly from the site of injection. However, significantly more radioactivity is recoverable from the body of PE\textsuperscript{32}P-treated than from that of \textsuperscript{32}P-treated animals. The difference is attributable to the accumulation of PE\textsuperscript{32}P at the site of injection, in the blood, liver and in the residual fraction. The radioactivity present in the liver and in the residual fraction of PE\textsuperscript{32}P-treated animals exhibited a very slow disappearance rate. On the other hand, the blood of these animals was cleared from radioactivity at a relatively rapid rate.

5. Evidence is presented suggesting that the bulk of radioactivity found in the liver and blood of PE\textsuperscript{32}P-treated animals is present in these organs as PE\textsuperscript{32}P.

6. Following a single intravenous injection, significantly more radioactivity is present in the body of PE\textsuperscript{32}P-treated animals, than in that of \textsuperscript{32}P-injected ones. This difference can be accounted for by the accumulation of PE\textsuperscript{32}P in the blood, liver, and residual fraction. Approximately half of the intravenously ad-
ministered PE\textsuperscript{32}P is taken up by the liver. Following intravenous administration, PE\textsuperscript{32}P disappears very slowly from the liver, spleen and residual fraction, whereas it is cleared from the blood at a relatively rapid rate.

7. Significantly more PE\textsuperscript{32}P is accumulated in the organism following intravenous than after subcutaneous administration. The difference can largely be attributed to the incorporation of increased amounts of PE\textsuperscript{32}P into the liver.

8. Similarly to the findings with PE\textsuperscript{32}P, significantly more \textsuperscript{32}P is retained in the organism following intravenous than after subcutaneous administration. This difference seems to depend on a general retention of intravenously administered \textsuperscript{32}P in the whole organism, rather than on an increased uptake by the liver.

9. Evidence is presented indicating, that the reticuloendothelial cell system is involved in the accumulation of PEP in the liver. A partial blockade of the reticuloendothelial system by india ink, or by PEP resulted in a reduced uptake of intravenously administered PE\textsuperscript{32}P by the liver. The diminished incorporation of PE\textsuperscript{32}P into the liver was paralleled by an increase in the blood concentration of this substance.

10. Evidence is presented, indicating that the prolonged duration of effectiveness of PEP does not depend on its accumulation in the liver; intravenous or intrasplenic administration of PEP – both of which lead to a great accumulation of PEP in the liver – resulted in a reduced duration of vaginal oestrus in spayed mice as compared to the effect elicited by subcutaneous administration.

11. Data are presented suggesting that a relationship may exist between circulating blood levels of PEP and duration of prolonged oestrogenic effect.

It is suggested that the high blood level of PEP, maintained during a relatively long period of time, may result in prolonged vaginal oestrus in spayed mice treated with this long-acting oestrogen.

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