laries or Thebesian veins thereby simulating AV transfers that may not exist.

Use of the heart-lung preparation precludes passage of microspheres into channels other than the pulmonary artery, and assures the propulsion of particles into the vasculature under physiological pressures. The recovery of spheres of 75-80 μ in the pulmonary venous blood leaves little doubt of the possibility of arteriovenous anastomoses in the pulmonary vasculature. Presumably these anastomotic connections function under conditions of increased pulmonary arterial pressure (such as occur in pulmonary embolism or heart failure) to spare the alveolar capillaries from overload. Other implied functions of these pulmonary AV shunts, as well as discrepant information as to their size, require further investigation.

Summary. Lucite spheres of 75-80 μ introduced into the pulmonary arteries of heart-lung preparations under physiologic pressures will pass from pulmonary arteries to veins. In contrast, microspheres of 300 μ size did not pass through the pulmonary vasculature.

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Influence of Varying Proportions in Combinations of Estrone, Estradiol and Estriol on Biological Response. (24027)

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Advances in analytical procedures, such as countercurrent distribution, as well as column and paper chromatography, have made it possible to identify and isolate estrogenic metabolites in relatively small (24-48 hour) collections of urine and in turn have stimulated much research. As a result, the interaction of these metabolites from a biological standpoint becomes even more intriguing. Hisaw and coworkers(1) and Wicks and Segal(2) stressed time and dose relationships. The concept of "impeded estrogens" was presented by Huggins and Jensen(3) and Edgren(4,5). Our interest in biological effectiveness of combinations of steroid hormones stems from 1948 when we reported results of androgenic mixtures on biological response(6). The present study was initiated in 1949.† Our aim was to see by what combination a synergistic effect could be realized or, if there was an inhibitory effect, at what level and in what combination it would be observed. From such data it may be possible to determine the type of biological activity that can be correlated with chemical assays, which determine presence and quantity of each estrogen in urine. A similar concept was postulated by Brown(7). Reports dealing with interaction of estrogen mixtures indicate that some workers employed either spayed mature(1) or hypophysectomized immature rats(3). Others employed intact immature mice(2,4,5). Other variables consisted of dose ranges, combination of estrogens and method of administering the mixtures.

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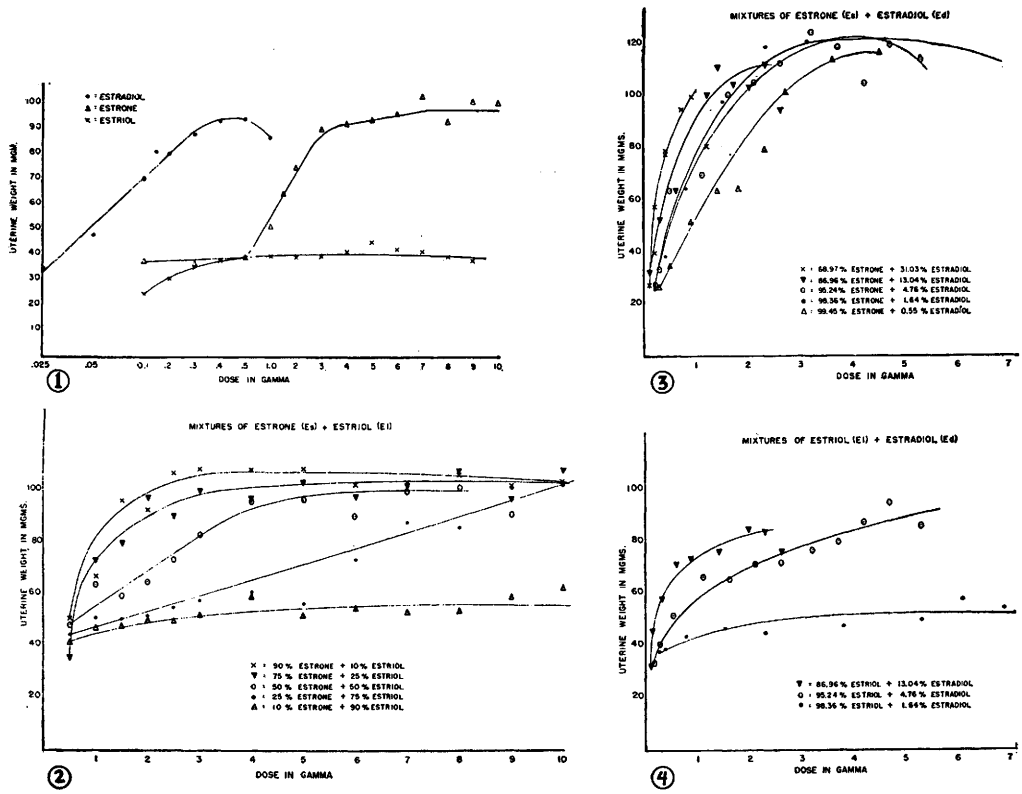

FIG. 1. Basic dose response curves established for estradiol 17 β, estrone and estriol 16 α, 17 β by uterine wt method in immature rats.

FIG. 2. Synergism is observed in the first 3 mixtures of the combination of the relatively biologically inactive estriol 16 α, 17 β with estrone. There is no inhibition of estrone by estriol 16 α, 17 β in any of the dose ranges in these mixtures.

FIG. 3. Inhibition appears in all combinations in the lowest dose range. Synergism occurs, depending upon proportions present in last 2 mixtures.

FIG. 4. Synergism is present in the very low dose range. Inhibition occurs only in the last mixture when estriol 16 α, 17 β portion was greater than 1.5 γ.

These variables tend to militate against valid comparisons of results obtained by different workers. We felt that simplification, by employing estrone as a standard for comparison with estradiol and with estriol in varying combination of mixtures, would be desirable. We chose the immature rat as test animal to eliminate response of target organ to preliminary stimulation or regression.

Methods and material. 2414 twenty-four day old immature rats of the Sherman strain were used. We employed 650 rats to establish basic dose response curves for the 3 estrogens studied (Fig. 1). 1613 animals were used to obtain data on mixtures that were studied and an additional 144 control animals were employed during the testing procedures. The rats were 24 days of age at time of first injection. Various doses of estriol, estradiol, and of estrone and combinations of these were dissolved in 0.6 ml of sesame oil and injected subcutaneously into the dorsum of each rat for 3 successive days. Rats were sacrificed on morning of fourth day. Body weights were recorded and the uteri removed and weighed after expressing the fluid. The ratio of body weight to uterine weight was calculated for a number of animals and was relatively constant. Therefore, only uterine weights were used to plot the curves. First we sought to establish the basic dose-response curves for estrone, estradiol 17β and estriol 16α, 17β (Fig. 1) using the uterine weight method. Relative potency of estriol and of estradiol

with estrone was then compared by determining equation of the line using the method of "least squares" and comparing the slopes. Previously(6) we combined biologically active androsterone with the much less active dehydroisoandrosterone, and achieved striking biological enhancement. We decided to use the same mixtures if possible for combinations of estrone and estriol. The mixtures (Fig. 2) were therefore made on weight basis and total dose range for each mixture was 0.5 to 10.0 γ. The mixtures were as follows: 90% estrone - 10 estriol, 75 - 25, 50 - 50, 25 - 75, 10 - 90%. In making mixtures wherein the very biologically active estradiol was to be combined with estrone in one case (Fig. 3) and with estriol in the other (Fig. 4), it was necessary to consider the fact that estradiol was approximately 20 times as active biologically as estrone. We wished, therefore, to keep the estradiol content in mixtures containing it on an equivalent biological basis with those set up for estrone and estriol. This was done as follows: *Mixture I* was made by combining 1 part of estradiol with 180 parts of estrone, which results in a mixture containing 99.45% by weight of estrone and 0.55% by weight of estradiol. On a biological basis, however, estradiol being 20 times as active as estrone, the above becomes biologically equivalent to 20 parts of estradiol combined with 180 parts of estrone or in a ratio, 1 part of estradiol to 9 parts of estrone. The other mixtures were made up as follows: *Mixture II*: 60 parts estrone + 1 part estradiol or 98.36% + 1.64%. Biologically: 60 parts estrone + 20 parts estradiol. *Mixture III*: 20 parts estrone + 1 part estradiol or 95.24% + 4.76%. Biologically: 20 parts estrone + 20 parts estradiol. *Mixture IV*: 20 parts estrone + 3 parts estradiol or 86.96% + 13.04%. Biologically: 20 parts estrone + 60 parts estradiol. *Mixture V*: 20 parts estrone + 9 parts estradiol or 68.97% + 31.03%. Biologically: 20 parts estrone + 180 parts estradiol. The mixtures for estradiol and estriol were made in exactly the same manner as the above. Estradiol combined in this manner results in dosages that in all cases are lower than or approximately the dose level required to achieve its maximum response.

Results. Reference to the basic dose response curve (Fig. 1) showed that estriol was the least active of the estrogens in promoting uterine growth and that estradiol was the most potent. A linear response could be secured for estrone up to a 3γ dose, for estradiol up to 0.15γ dose, and for estriol up to a 0.4γ dose. Beyond these doses there is a leveling off of biological response. The equations derived from the dose response data for each of the 3 estrogens in their respective dose ranges are as follows:

Estrone	.1γ to 3.0γ	$y = 23.3x + 29.0$
Estriol	.1γ to .4γ	$y = 42.3x + 21.9$
Estradiol	.02γ to .15γ	$y = 404.1x + 24.8$

By comparing the slopes of these equations it can be seen that estradiol is 17.4 times as effective as estrone. This value is not too far removed from the 20:1 ratio observed by other workers (Lauson, 8). We found, as did Lauson(8) that estriol was *more* potent than estrone *in the very low dose range* (*below 0.4γ*). Our experiments showed the significant fact that estriol is 1.8 times as potent as estrone at this level (cf. Discussion).

Comparisons within the 3 dose response curves hold only for the limits of effectiveness of the compared individual estrogens. It does not follow that 1γ of estradiol when injected into an animal will be equivalent in effect to 17.4γ of estrone, since the peak of biological response as far as estrone is concerned is reached at 4γ. Any further increase in dosage results in a leveling off or plateau as shown in the curves. It will be shown later that it is only when mixtures of the various estrogens are used that it is possible to exceed this peak.

On the basis of these responses we were interested in what effect the combination of a fairly biologically inert estrogen, such as estriol with estrone alone and with estradiol alone would have. The second part of the experimental study deals with these mixtures and the results that were obtained.

The results of the study of these varying proportions of mixtures of estrone + estradiol; estrone + estriol; and of estriol + estradiol show that each combination exhibits behavior peculiar to that pair of estrogens.

The results obtained with each combination are therefore described separately as follows:

Estrone + estriol (Fig. 2). The combination of the relatively inactive estriol with estrone resulted in the most striking synergistic effects. In the mixtures of 90% estrone with 10% estriol, 75% - 25%, and 50% - 50% synergism was observed in the total dose range from 1 to 5γ. Beyond the total of 5γ no synergism was observed but neither was there an inhibitory influence of estriol on estrone. Increments of estriol from 0.05γ to 5.00γ when mixed with estrone in the above mentioned proportions produced synergism. In the mixture of 25% estrone with 75% estriol synergism was attained only in the highest dose range, and not at all in the mixture of 10% estrone with 90% estriol. It is significant also that estriol did not inhibit the estrone activity *per se* in any of the mixtures. Reference to the curves for the respective mixtures of the estrogens and to the basic dose response curves was made in order to determine whether or not synergism‡ had occurred. This determination was made as follows: The response§ to a dose for a given mixture was read from the curve of that mixture. This value was then compared to the sum of the responses for the constituents of this dose as determined from the individual basic dose response curves. This procedure was used to interpret all other combinations of the estrogens.

Estrone + estradiol (Fig. 3). Mixtures of varying proportions of estrone with estradiol (Fig. 3) revealed a distinctly different pattern from those when the relatively inactive estriol was combined with either one of these more potent estrogens (Fig. 2-4). In the very low dose range for each mixture the combination of estrone and estradiol resulted in an inhibitory effect on uterine stimulation. This was almost akin to antagonism. As the dose was increased, synergism was produced in a varying degree dependent upon the proportion in which the 2 estrogens were combined. In the first mixture, wherein 99.45% by weight of estrone was combined with 0.55% by weight of estradiol, synergism was achieved in the total dose range from 0.905 to 4.525γ. In the mixture consisting of 98.36% by weight of estrone and 1.64% by weight of estradiol, synergism was achieved in the total dose range from 0.381 to 4.58γ. A tenuous enhancement was achieved for a very narrow dose range in the next 2 mixtures. It is interesting to note that the peak of biological responsiveness for these mixtures was at a higher uterine weight level than the peak for either estrone or estradiol alone. We can summarize our observations as to the effect of mixtures of estrone and estradiol on the immature rat uteri as follows: There is a dose range which results in an inhibition of uterine growth followed by a dose range which results in stimulation of this growth to a point of synergism and a dose range beyond which the animal cannot respond—a so-called point of non-responsiveness.

Estriol + estradiol (Fig. 4). The results obtained with mixtures of estriol and estradiol showed that here again it was possible to achieve synergism but only in the very low dose range of the 3 mixtures in this combination. In the mixture consisting of 98.36% estriol and 1.64% estradiol, we found evidence for inhibition of estradiol by estriol when the estriol portion of the total dose was greater than 1.5γ. In the other 2 mixtures, however, estriol did not inhibit the effect of estradiol even after its portion of the total dose had gone over 1.5γ. It is realized that a dose of 1.5γ of estriol is far beyond the maximum biological response dosage for estriol but this was the only region where inhibition was manifest.

Discussion. The synergistic effect which was obtained with mixtures in the low dose range was a fairly consistent observation in all of the combinations that were studied with the exception of the estrone + estradiol mixture. In this combination an inhibitory effect on uterine stimulation almost akin to an-

‡ The term synergism is employed only when uterine response to the dose of the estrogenic mixtures is greater than or equal to the sum of the individual uterine responses for each constituent of the mixture.

§ The term response is used for net uterine weight, i.e. data corrected for control weight.

tagonism was observed in the very low dose range.

In specific instances there is an enhancing influence by the almost biologically inactive estriol. This points up an important consideration in the investigation of the influence of minute amounts (up to 0.4γ) of presumably inactive hormones in maintenance of hormonal balance. The most striking effect of this study of mixtures is the behavior of estrone + estriol combinations where synergism was observed up to the 5γ dose level for 3 out of 5 mixtures. It was likewise significant that estriol did not inhibit estrone activity in any of the mixtures. The potentiation resulting from mixtures is unexplained. Whether it occurs at the target tissue level or as the result of chemical changes of the compounds by the other tissues of the test animal is to be pursued. It is conceivable, however, that with the combination of estrone and estradiol, a mass action effect takes place *in vivo*. Presumably the estrone retards the conversion of the estradiol to estrone and thus increases the net estradiol actually present in the mixture at target organ level to create the effect we observed as synergism.

The synergistic effects observed with estrone + estriol may be due to a block of an active degradation site of the estrone to estriol thus increasing the estrone-estradiol interchange. The possible influence of the presence of estriol during the presumed conversion of estrone to estradiol is to be developed. On the other hand, when combinations of estrone and estriol reach a dose level beyond the peak of response for estrone alone, there is a decrease in response which may be accounted for by a biological reaction which disturbs hormonal equilibrium or, it may be accounted for by a lessening of activity due to a competitive action by both of these estrogens for the active sites of the target organ.

The explanation for the inhibition of estradiol by estriol in the 98.36% estriol + 1.64% estradiol may also be due to competitive action by these 2 estrogens for the target organ. In this mixture the amount of estriol present is considerably beyond the limit of its biological effectiveness. In competition for active sites in the target organ it may overwhelm the amount of estradiol present. In the other mixtures in this series the amount of estradiol is of such proportion that as it approaches its peak of response it is able to counteract the effect of the estriol at the tissue level.

An explanation of the synergism exhibited by estradiol and estriol in the low dose range may be on the same basis as the suggested mechanism at work when estrone was combined with estriol. Or, it could be speculated that at the low dose level there is no competition for the target organ and that a summation of effect is being attained.

Summary. Basic dose response curves for estrone, estradiol, and estriol were established using uterine weight method in immature female rats of the Sherman strain. Combinations of estrone + estriol; estrone + estradiol; and estradiol + estriol were set up to study their effect on stimulation of the immature rat uterus. The interpretations were based on the data of dose response curves. A synergistic effect was observed in all of the mixtures studied in the low dose level except with the mixture of estrone + estradiol, which showed it in the higher dose levels. A combination of estrone + estriol resulted in synergism being observed for 3 out of the 5 mixtures studied up to a 5γ dose level. Estriol did not inhibit estrone activity in any of the mixtures. In the study of the 3 estradiol + estriol mixtures there was evidence of inhibition of estradiol by estriol in only one of the mixtures. The manner of action of these combinations of estrogens has been postulated.

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