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ACTIVITY OF THE RETICULOENDOTHELIAL SYSTEM AND THE ANTIBODY RESPONSE

I. EFFECT OF STILBOESTROL ON RES ACTIVITY AND LOCALIZATION OF SHEEP ERYTHROCYTES IN THE MOUSE

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Summary.—The stimulatory effect of stilboestrol on the RES of the mouse was assessed in terms of the blood clearance and organ uptake of colloidal carbon, sheep RBC and heat damaged mouse RBC, and comparison made between the effect on the RES of stilboestrol and endotoxin. Pretreatment with stilboestrol caused an increase of hepatic uptake of test materials and a reduction of carbon and SRBC localization in the spleen. Incorporation of [^{3}H]thymidine and the proportion of labelled cells were increased in livers and spleens of stilboestrol treated animals.

THE phagocytic activity of the reticuloendothelial system (RES) in intact animals is usually assessed by measuring the rate of blood clearance of particulate materials (Benacerraf, 1964; Biozzi and Stiffel, 1965). It is well known that a variety of substances can either enhance or depress blood clearance rates, which is generally interpreted as indicating increased or reduced phagocytic capacity of RE cells (Stiffel, Mouton and Biozzi, 1970). In the majority of studies information on the organ distribution of the injected material is lacking, partly because of the difficulty of quantitative measurement in tissues of colloidal carbon, a substance which is commonly used for clearance studies (Šljivić, 1969).

Oestrogens, both natural and synthetic, are recognized stimulants of the RES (cf. Vernon-Roberts, 1969) and among them stilboestrol is one of the most potent (Nicol *et al.*, 1963). These hormones greatly enhance blood clearance rates and increase the number of phagocytic cells in the liver (Kelly, Brown and Dobson, 1962). There are very few studies, however, of the effect of oestrogens on the organ distribution of injected particulate materials, including antigens. Such information might be expected to contribute to the understanding of the mode of action of oestrogens on the RES and perhaps to throw some light on their controversial effects on antibody responses (Šljivić and Warr, 1973*a*).

An evaluation of the stilboestrol induced alteration of the phagocytic activity of the RES with respect to colloidal carbon and red blood cells is given in the present paper. The accompanying paper (Šljivić and Warr, 1973*b*) deals with the effect of stilboestrol on the immune response to SRBC.

MATERIALS AND METHODS

Animals.—Female CBA mice were used for most experiments. They were 3–4 months old and weighed 18–24 g. A few experiments were performed on male CBA/H and outbred female AS1 mice.

RES stimulation.—Stilboestrol (diethylstilboestrol) was injected s.c. at the dose of 1 mg in 0·2 ml arachis oil. In some experiments control animals injected with oil alone were included.

Endotoxin was dissolved in saline and injected i.v. in a volume of 0·2 ml. Two preparations of lipopolysaccharides were used: *E. coli* 0111 : B4 (Difco Laboratories, East Molesey, Surrey, England) and *E. coli* 07.K1.H6 prepared by the Westphal's phenol–water method (we are indebted to Professor A. A. Glynn of the Bacteriology Department, St Mary's Hospital Medical School, for a gift of this material).

Colloidal carbon.—Colloidal carbon (C11/1431a, Günther Wagner, Pelikan Werke, Hanover, Germany) was diluted with 1% gelatin solution as described before (Šljivić, 1970), and injected i.v. *via* a tail vein. For clearance studies an adjusted dose of carbon was injected (0·01 ml/g body weight of a 1 in 5·625 dilution which corresponds to approximately 16 mg/100 g body weight) while for uptake studies both a standard dose of 50 μl original carbon suspension diluted to 0·3 ml with gelatin (Šljivić, 1969) and an adjusted dose were used.

^{51}Cr-labelled red blood cells.—Sheep red blood cells (SRBC) in Alsever's solution (Tissue Culture Services Ltd, Slough, England) were washed with sterile normal saline and a suitable amount of ^{51}Cr (Sodium Chromate *B.P.*, Radiochemical Centre, Amersham) was added taking care not to exceed 5 μg Cr per ml of packed cells. After incubation at room temperature for approximately 30 min the cells were washed 3 times in sterile saline, resuspended at the required concentration, and injected in a volume of 0·25 ml. Syngeneic mouse red blood cells (MRBC) from fresh heparinized blood were labelled with ^{51}Cr as above. They were then damaged by heating at 49·5° for 10 min, washed twice more and 4 × 10^8 cells in 0·2 ml were injected into mice i.v.

Clearance studies.—The rate of clearance of colloidal carbon was measured as described before (Šljivić, 1970) and the constant was calculated from $K = 0{\cdot}693/t_{\frac{1}{2}}$. Clearance of ^{51}Cr-labelled SRBC and MRBC was measured in a similar way by taking 4–5 10 μl aliquots of blood (drawn with heparinized Microcap disposable micropipettes, Shandon Scientific Co. Ltd) from the orbital venous plexus of mice under light ether anaesthesia. These blood samples were diluted in 1 ml of saline and their radioactivity measured in a Packard well-type scintillation counter.

Organ uptake.—At selected intervals after the injection of colloidal carbon or RBC mice were killed by cervical dislocation. Organs were removed and livers washed with saline and blotted with filter paper. The amount of colloidal carbon present in the organs 24 hours after injection was determined by a quantitative spectrophotometric method (Šljivić, 1969). For organ distribution of ^{51}Cr-labelled RBC, radioactivity of whole organs was measured in the scintillation counter and expressed as percentage of the dose injected. In the case of SRBC, uptake at 15 min after injection was taken to reflect adequately the relative phagocytic activity of the liver and spleen, while the radioactivity at 24 hours after injection was used to indicate the final organ distribution.

Incorporation of [^{3}H]thymidine.—Animals received three intraperitoneal injections of 25 μCi [^{3}H]thymidine (methyl[^{3}H]thymidine, 18 Ci/mmol, Radiochemical Centre, Amersham) in 0·1 ml normal saline at 2-hourly intervals. They were killed by cervical dislocation 2 hours after the last injection. Weighed portions of the liver weie homogenized in 5 ml normal saline. Mesenteric lymph nodes, bone marrow from one femur, and portions of the thymus and spleen were sieved into 2·0 ml Tyrode's solution to produce cell suspensions. Portions of these cell suspensions were taken to determine nucleated cell counts. Duplicate aliquots of liver homogenate and spleen, thymus, bone marrow and mesenteric lymph node cell suspensions were precipitated in ice-cold 10% trichloracetic acid (TCA), centrifuged at 400 *g*, and washed once in ice-cold 5% TCA and twice in ice-cold absolute ethyl alcohol. Each precipitate was dissolved in 0·2 ml hyamine hydroxide (Nuclear Enterprises Ltd, Sighthill, Edinburgh) at 60° for 5 min with constant shaking and added to 4 ml of scintillation fluid (Instagel, Packard Instrument Ltd, London) for counting in a Packard TriCarb scintillation counter.

Autoradiography and histology.—Sternum, inguinal lymph nodes, and portions of liver, spleen and thymus from mice injected with [^{3}H]thymidine were fixed for 1 hour in acetic acid-alcohol (1 : 3 by volume) followed by 24 hours in 10% formol saline, embedded in paraffin wax and cut at 5 μm. Autoradiographs were prepared by the stripping-film technique using Kodak AR. 10 film, exposed for 3 weeks, developed (Kodak, D-19) and stained with haematoxylin and eosin. The percentage of labelled cell nuclei in liver sections was determined by counting over 2000 nuclei for control animals and about 1000 nuclei for stilboestrol injected animals using a $\times 100$ objective and with the help of a graticule.

For histology of colloidal carbon localization mice were killed 3–4 hours after carbon injection. Livers and spleen were fixed in Bouin's fluid, cut at 5 μm and stained with haematoxylin and eosin. Unstained sections were also prepared and used to examine carbon distribution and for microphotography.

Serum transfer.—Fresh serum from untreated CBA mice and from CBA mice which had received stilboestrol 3 days previously was obtained and 0·4 ml injected i.v. into syngeneic recipients. A test dose of 5×10^{8} ^{51}Cr-labelled SRBC was injected i.v. 90 min later to determine the organ distribution.

Statistics.—Student/Welch's *t*-test (Cochran and Cox, 1957) was used to analyse the significance of differences between experimental and control groups.

RESULTS

Clearance and distribution of colloidal carbon

The rates of blood clearance and amounts of colloidal carbon in spleens and livers of mice pretreated with stilboestrol are given in Table I. The results obtained in mice injected with *E. coli* endotoxin, another well established stimulant of the RES, are included for comparison. The rate of blood clearance increased gradually from the first day after injection of stilboestrol and on Day 3 was comparable to that found in mice injected with endotoxin. The results of the 2 separate organ uptake experiments indicate that the distribution of colloidal carbon was differently affected by the 2 RES stimulants used. The splenic uptake in stilboestrol treated mice tended to be lower than in untreated controls, whereas in animals treated with endotoxin it was unaltered or increased. The hepatic uptake was increased in mice treated with stilboestrol but not in those injected with endotoxin.

Histological examination revealed changes in tissue localization of colloidal carbon in mice pretreated with stilboestrol. The amount of carbon present in bone marrow sections was very much reduced or even undetectable compared with control animals. In the spleens of control mice carbon was generally uniformly distributed throughout the red pulp. After treatment with stilboestrol the amount of carbon was greatly reduced in the red pulp but concentrated in a characteristic fashion in the marginal zone surrounding the Malpighian bodies (Fig. 1). A similar pattern of carbon localization could be found in untreated mice injected with a smaller amount of colloidal carbon. Administration of endotoxin did not result in such an alteration of carbon localization (Fig. 1).

Clearance and distribution of ^{51}Cr-labelled SRBC

In the study of the distribution of ^{51}Cr-labelled SRBC between the liver and the spleen 2 endpoints were used. The first was the uptake 15 min after the injection of a saturating dose of SRBC, this being found suitable, because of the relatively low liver uptake, for the assessment of RES stimulation after administration of stilboestrol or other agents. The other was uptake at 24 hours, this representing the final distribution of SRBC between the 2 major sites of uptake.

EXPLANATION OF PLATES

FIG. 1.—Distribution of colloidal carbon in mouse spleens: (A) control, (B) 3 days after stilboestrol (C) 3 days after endotoxin 0111 : B4, (D) control injected with 25 μl of colloidal carbon (A–C received 50 μl). Unstained sections. $\times 110$. WP—white pulp, RP—red pulp.

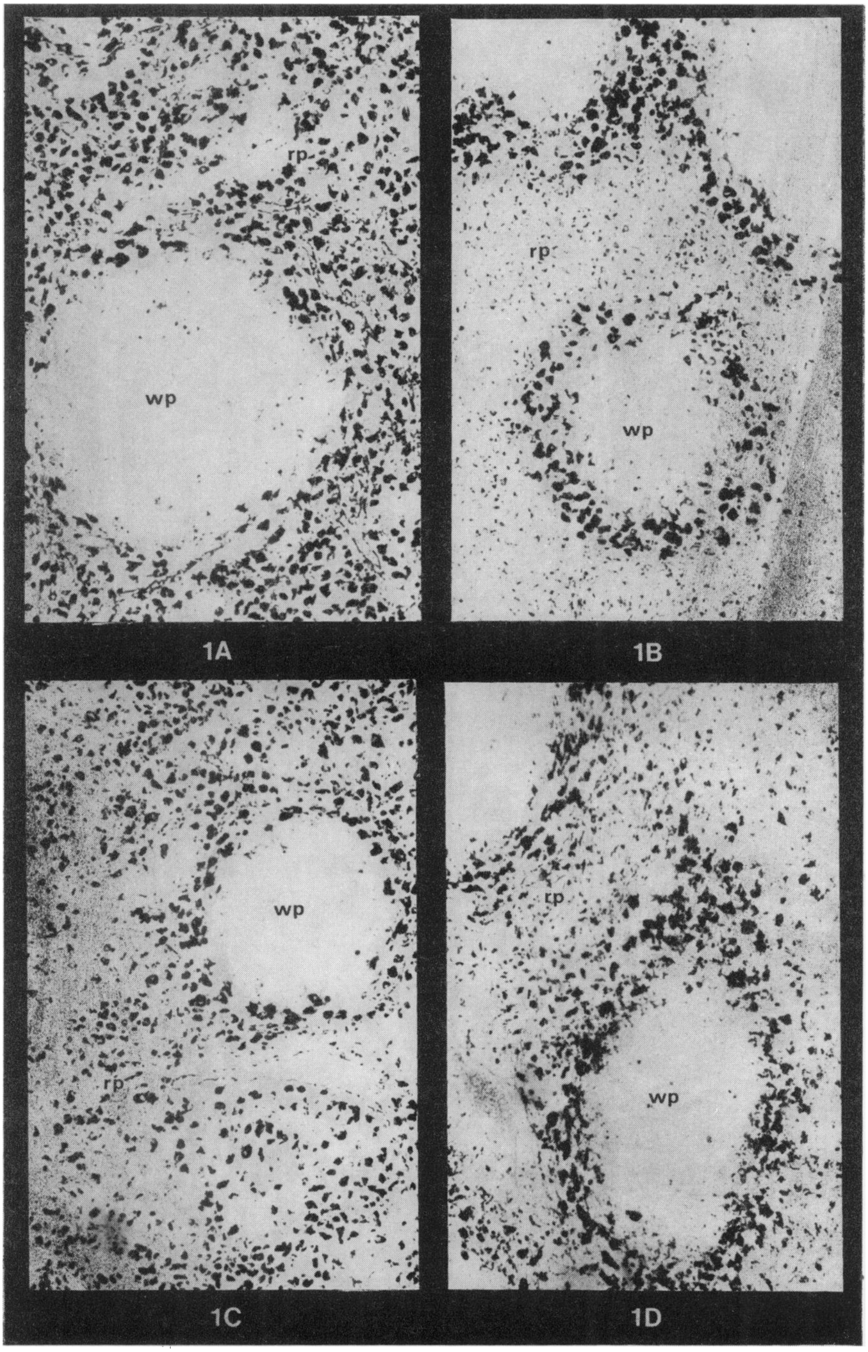

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TABLE I.—*Effect of Stilboestrol and Endotoxin on the Rate of Clearance and Organ Uptake of Colloidal Carbon*[a]

Treatment	Days after treatment	Rate of clearance ($K \times 100$)*	Liver: Weight g/100 g body weight	Liver: Percentage uptake[b]	Spleen: Weight (mg)	Spleen: Percentage uptake[b]	Number of mice
Untreated controls	–	$3 \cdot 02 \pm 0 \cdot 18$	$5 \cdot 40 \pm 0 \cdot 16$	$52 \cdot 1 \pm 1 \cdot 7$	90 ± 4	$10 \cdot 40 \pm 0 \cdot 75$	5
Stilboestrol	1	$3 \cdot 71 \pm 0 \cdot 14^{c}$	N.D.	N.D.	N.D.	N.D.	5
Stilboestrol	2	$5 \cdot 59 \pm 0 \cdot 37^{c}$	N.D.	N.D.	N.D.	N.D.	5
Stilboestrol	3	$8 \cdot 72 \pm 0 \cdot 35^{c}$	$6 \cdot 61 \pm 0 \cdot 21^{c}$	$65 \cdot 7 \pm 1 \cdot 3^{c}$	118 ± 10^{c}	$8 \cdot 00 \pm 0 \cdot 68^{c}$	5
Endotoxin[d]	3	$7 \cdot 32 \pm 0 \cdot 39^{c}$	$5 \cdot 66 \pm 0 \cdot 07$	$56 \cdot 5 \pm 2 \cdot 0$	136 ± 7^{c}	$7 \cdot 97 \pm 1 \cdot 03$	5
Untreated controls	–	N.D.	$6 \cdot 35 \pm 0 \cdot 14$	$75 \cdot 9 \pm 0 \cdot 9$	115 ± 4	$10 \cdot 45 \pm 0 \cdot 19$	10
Stilboestrol	3	N.D.	$7 \cdot 07 \pm 0 \cdot 23^{c}$	$86 \cdot 0 \pm 1 \cdot 2^{c}$	103 ± 5^{c}	$6 \cdot 98 \pm 0 \cdot 48^{c}$	5
Endotoxin[e]	3	N.D.	$6 \cdot 67 \pm 0 \cdot 11$	$65 \cdot 5 \pm 2 \cdot 8^{c}$	156 ± 6^{c}	$18 \cdot 29 \pm 2 \cdot 03^{c}$	5

(a) Results of 3 separate experiments are shown. Mice used in the upper part of the table were CBA females and were injected i.v. with a dose of colloidal carbon adjusted to body weight (see under *Materials and Methods*). Mice in the lower part of the table were CBA males and these were injected with a standard dose of carbon. Values are means ± s.e.
(b) Percentage of the dose injected.
(c) Significantly different from untreated controls ($P < 0 \cdot 05$ to $P < 0 \cdot 001$).
(d) 50 μg of *E. coli* endotoxin 07.K1.H6.
(e) 200 μg endotoxin 0111 : B4.
* The rates of clearance were determined on separate groups of mice.

The effect of stilboestrol on the rate of clearance and uptake in the liver and spleen of ^{51}Cr-labelled SRBC is shown in Table II, and it can be compared with the effect produced by endotoxin. Although both these agents greatly enhanced the rate of blood clearance of SRBC, and markedly increased the liver uptake, their effect on the splenic uptake was fundamentally different. While endotoxin increased the splenic uptake of SRBC, pretreatment with stilboestrol caused a marked reduction. This differential effect is probably best illustrated by the liver/spleen ratios (Table II). That this effect is due to stilboestrol and not the

TABLE II.—*Comparison of the Effects of Stilboestrol and Endotoxin on the Blood Clearance and Organ Distribution of ^{51}Cr-Labelled SRBC*[a]

Treatment	Rate of clearance* ($K \times 100$)	Percentage uptake[b] Liver	Spleen	Liver/spleen ratio
Untreated controls	$4 \cdot 24 \pm 0 \cdot 55$	$33 \cdot 6 \pm 3 \cdot 46$	$7 \cdot 28 \pm 0 \cdot 51$	4·61
Stilboestrol[c]	$34 \cdot 68 \pm 3 \cdot 65$[d]	$96 \cdot 4 \pm 3 \cdot 61$[d]	$0 \cdot 87 \pm 0 \cdot 16$[d]	110·9
Endotoxin[c]	$22 \cdot 41 \pm 3 \cdot 54$[d]	$69 \cdot 5 \pm 3 \cdot 56$[d]	$12 \cdot 59 \pm 0 \cdot 77$[d]	5·52

(a) Values are means $\pm$ s.e. for 5 CBA mice in each group.
(b) Percentage of the dose given 15 min after an i.v. injection of 4×10^8 SRBC.
(c) Stilboestrol (1 mg s.c.) and endotoxin (07.K1.H6, 50 μg i.v.) given 3 days before.
(d) Significantly different from control values ($P < 0 \cdot 001$).
* Based on radioactivity of blood samples taken 3, 8 and 13 min after injection.

oil in which it was dissolved is shown in Fig. 2, which also indicates the changing pattern of liver and spleen uptake of SRBC during the first 4 days after administration of stilboestrol. The maximum divergence of SRBC uptake by these 2 organs

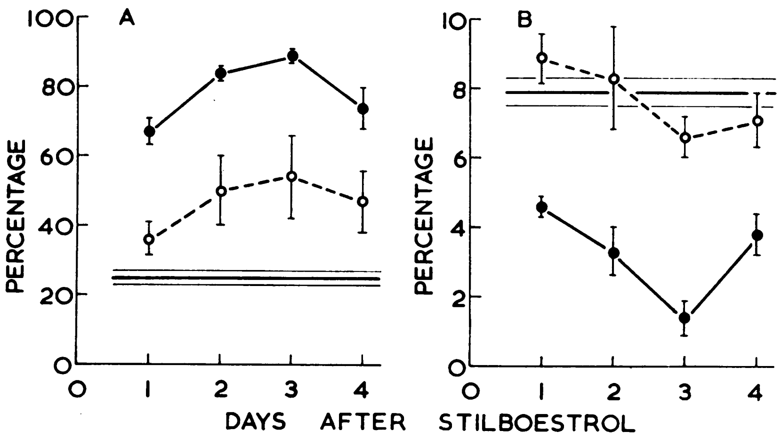

FIG. 2.—The 15-min uptake of ^{51}Cr-labelled SRBC by the liver (A) and spleen (B) at times after administration of stilboestrol (●) or arachis oil (○). Means with s.e. for 5 mice in each group are shown. The mean uptake with s.e. in untreated mice is shown by horizontal lines.

occurred 3 days after stilboestrol and then gradually decreased, but was still present 6 days later in another experiment not shown here.

The effect of stilboestrol on the organ distribution of SRBC could also be demonstrated in mice killed 24 hours after its injection. Results of an experiment

TABLE III.—*Effect of Stilboestrol, Dose of SRBC and Route of Injection on the Localization of* ^{51}*Cr-Labelled SRBC*[a]

Treatment: Dose of SRBC	Treatment: Route	Treatment: Stilboestrol[b]	Liver: Weight g/100 g body weight	Liver: Percentage uptake[c]	Spleen: Weight (mg)	Spleen: Percentage uptake[c]	Liver/spleen ratio	Number of mice in each group
10^7	i.v.	−	5·57±0·25	86·7±2·9	82±1	2·51±0·25	34·54	4
10^7	i.v.	+	6·11±0·40	88·2±1·9	91±7	0·45±0·29[d]	196·00	4
10^8	i.v.	−	5·37±0·25	72·9±1·9	99±4	7·87±0·44	9·26	4
10^8	i.v.	+	6·26±0·26[d]	92·7±2·1[d]	89±7	0·85±0·24[d]	109·06	4
10^9	i.v.	−	5·15±0·12	13·7±1·5	95±6	12·80±0·60	1·07	4
10^9	i.v.	+	6·40±0·11[d]	66·0±5·0[d]	111±7	3·97±0·89[d]	16·62	4
10^7	i.p.	−	5·54±0·23	8·0±1·2	61±5	0·41±0·12	19·61	8
10^7	i.p.	+	6·05±0·31	9·6±0·6	69±8	0·36±0·04	26·64	4
10^8	i.p.	−	5·17±0·16	35·3±4·4	83±9	2·58±0·28	13·68	4
10^8	i.p.	+	6·15±0·42[d]	28·3±4·9	85±11	1·53±0·11[d]	18·48	4
10^9	i.p.	−	5·43±0·23	26·4±1·2	90±9	12·10±2·51	2·19	4
10^9	i.p.	+	6·36±0·17[d]	54·9±6·9[d]	87±6	1·32±0·07[d]	41·58	4

(a) All values are means ± s.e. for groups of CBA mice.
(b) Stilboestrol was given 3 days before injection of SRBC.
(c) Percentage of the dose given 24 hours after injection of labelled SRBC.
(d) Significantly different from untreated controls ($P < 0 \cdot 05$ to $P < 0 \cdot 001$).

TABLE IV.—*Effect of Stilboestrol and the Dose of SRBC on the Localization of ^{51}Cr-Labelled SRBC in Primed Mice*[a]

Treatment			Liver		Spleen		
Dose of SRBC	Route	Stilboestrol[b]	Weight g/100 g body weight	Percentage uptake[c]	Weight (mg)	Percentage uptake[c]	Liver/spleen ratio
10^6	i.v.	−	e	19·9±1·6	81±3	1·31±0·45	15·19
10^6	i.v.	+	6·95±0·58	22·9±0·8	80±4	0·68±0·56	33·68
10^7	i.v.	−	5·43±0·04	40·1±0·9	88±5	0·94±0·06	42·66
10^7	i.v.	+	6·47±0·20[d]	45·5±1·0[d]	107±9[d]	0·32±0·07[d]	142·19
10^9	i.v.	−	5·22±0·15	10·1±0·7	85±5	4·58±0·78	2·21
10^9	i.v.	+	6·14±0·10[d]	41·0±9·4[d]	116±6[d]	2·60±0·33[d]	15·77

(a) CBA mice were sensitized with SRBC 60 days before the test. All values are means ± s.e. for 4 mice in each group.
(b) Stilboestrol was given 3 days before injection of SRBC.
(c) Percentage of the dose given 24 hours after injection of labelled SRBC.
(d) Significantly different from untreated controls ($P < 0·05$ to $P < 0·001$).
(e) Data missing.

performed in CBA mice which had received stilboestrol 3 days previously are shown in Table III. The splenic uptake was reduced at all doses of SRBC whether injected i.p. or i.v.; liver uptake, on the other hand, was not substantially altered after the 2 lower doses of SRBC (10^7 and 10^8) but increased in stilboestrol treated mice after 10^9 SRBC. The change of the relative liver and spleen uptake is again indicated by the ratio between the 2 organs. It can be noted, too, that the organ uptake for the same dose of SRBC was generally higher after an i.v. injection than after an i.p.. one, except for the dose of 10^9 SRBC. A very similar pattern of organ distribution over a range from 10^6 to 10^9 SRBC injected i.v. and i.p. was found in AS1 mice.

Pretreatment with stilboestrol also affected the organ distribution of SRBC in mice which had been primed with the same antigen 60 days previously (Table IV). The splenic uptake was again reduced as a result of stilboestrol administration. The percentage organ uptake for the same dose of SRBC was generally lower in primed than in unprimed animals, presumably because of intravascular lysis.

In order to test the extent to which the reduced splenic uptake of SRBC is the result of increased liver activity, a dose of colloidal carbon sufficient to block liver uptake in control animals was injected 6 hours before SRBC (Souhami, 1972). Although the amount of carbon used was insufficient to block liver uptake in stilboestrol treated mice to the same degree as in control ones, splenic uptake was markedly increased compared with that in non-blockaded animals (Table V).

TABLE V.—*Effect of Stilboestrol and RES Blockade on SRBC Localization*

Treatment		Percentage uptake[a]		
Blockade[b]	Stilboestrol[c]	Liver	Spleen	Liver/spleen ratio
−	−	$88 \cdot 5 \pm 2 \cdot 9$	$4 \cdot 1 \pm 0 \cdot 7$	21·6
−	+	$92 \cdot 0 \pm 2 \cdot 2$	$0 \cdot 34 \pm 0 \cdot 1$	270·6
+	−	$4 \cdot 6 \pm 0 \cdot 8$	$23 \cdot 5 \pm 2 \cdot 5$	0·20
+	+	$43 \cdot 4 \pm 7 \cdot 3$	$15 \cdot 4 \pm 1 \cdot 9$	2·82

(a) Percentage of the dose given 24 hours after an i.v. injection of 10^8 ^{51}Cr-labelled SRBC. Values are means ± s.e. for groups of 5 CBA mice.
(b) Colloidal carbon (50 μl in 0·3 ml gelatine) injected i.v. 6 hours before SRBC.
(c) Stilboestrol given 3 days previously.

Serum transfer

Administration of oestrogens has been reported to increase serum γ-globulin levels (Nicol *et al.*, 1964) and natural heterophil haemagglutinins (Von Haam and Rosenfeld, 1942). A serum transfer experiment was performed in order to test the possibility that treatment with stilboestrol might alter some opsonic factor and thus result in a different organ distribution of SRBC. No difference could be detected in the liver and spleen uptake of ^{51}Cr-labelled SRBC between mice which had received serum from stilboestrol treated donors and those which received control serum.

Distribution of ^{51}Cr-labelled heat-damaged MRBC

In contrast to the findings with SRBC, the relative distribution of heat-damaged syngeneic RBC, which are preferentially taken up by the spleen, was little affected by RES stimulation with stilboestrol or endotoxin, as can be seen from the ratios of the uptake by the 2 organs (Table VI). Both treatments increased to a varying degree the liver and spleen uptake, except for the spleen uptake in the stilboestrol treated group which was unchanged.

TABLE VI.—*Liver and Spleen Distribution of ^{51}Cr-Labelled Heat-Damaged Syngeneic RBC in RES Stimulated Mice*[a]

Treatment	Percentage uptake[b] Liver	Spleen	Liver/spleen ratio
Untreated controls	$11\cdot6\pm1\cdot0$	$23\cdot3\pm1\cdot5$	0·50
Stilboestrol[c]	$16\cdot1\pm1\cdot6$[e]	$25\cdot0\pm2\cdot2$	0·64
Endotoxin[c]	$25\cdot4\pm1\cdot4$[e]	$30\cdot5\pm1\cdot1$[e]	0·83

(a) Values are means $\pm$ s.e. for 5 CBA mice in each group.
(b) Percentage of the dose given 4 hours after an i.v. injection of 4×10^8 MRBC.
(c) Stilboestrol (1 mg s.c.) and endotoxin (07.K1.H6 50 μg i.v.) given 3 days before.
(e) Significantly different from untreated controls ($P < 0\cdot05$ to $P < 0\cdot001$).

Incorporation of [³H]thymidine into liver and spleen

The incorporation of [³H]thymidine into various mouse tissues was studied during the first 3 days after administration of stilboestrol. The results obtained for the liver and the spleen are given here and those for other tissues are described in the accompanying paper (Šljivić and Warr, 1973*b*). As soon as one day after administration of stilboestrol there was an increase of thymidine incorporation in the liver and a relatively smaller increase in the spleen (Fig. 3). The maximal incorporation in both organs occurred on Day 2, after which it declined.

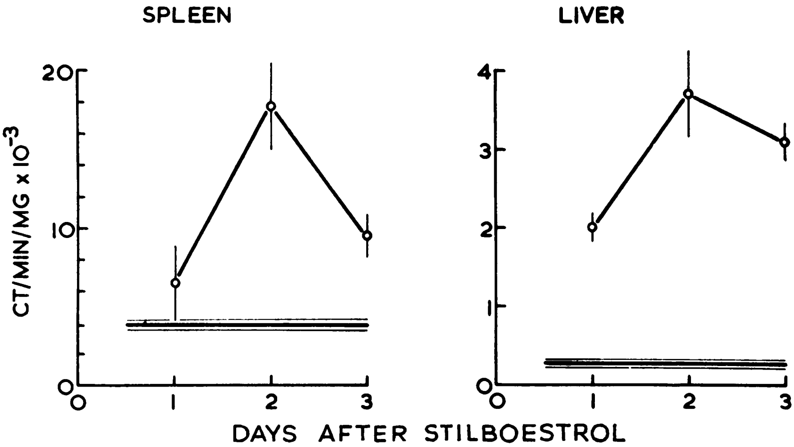

FIG. 3.—Incorporation of [³H]thymidine into the spleen and liver at times after administration of stilboestrol. Means with s.e. for 3 mice in each group are shown. The level in untreated control mice is indicated by horizontal lines.

The results of differential cell counts on autoradiographs of liver sections from the same experiment are shown in Table VII. The proportion of Küpffer cells in relation to the total number of cells in areas which were scored nearly doubled by Day 3 after stilboestrol. During the same period there was a rise in the percentage of labelled Küpffer cells, reaching a peak on Day 2, as well as some increase in the

TABLE VII.—*Proportion of Küpffer Cells and [^3H]thymidine Labelled Cells in Liver Sections of Control and Stilboestrol-treated Mice*[a]

Experimental group	Percentage Küpffer cells[b]	Percentage labelled cells	
		Küpffer cells	Parenchymal cells
Untreated control	37·6, 36·8, 32·0	0·57, 0·80, 0·93	0·11, 0, 0
1 day after stilboestrol	50·1, 44·6	30·2, 17·5	0·95, 0·30
2 days after stilboestrol	47·4, 42·3, 49·2	37·8, 33·0, 47·2	1·95, 0·99, 1·20
3 days after stilboestrol	60·3, 60·8, 47·5	31·6, 25·3, 30·6	2·30, 3·20, 0·64

(a) For schedule of [^{3}H]thymidine injection and numbers of cells scored see *Materials and Methods.* Each value was obtained from liver sections of one animal.
(b) Percentage of all nucleated cells counted.

percentage of labelled parenchymal cells. These changes are comparable to those reported by Kelly, Brown and Dobson (1962).

The labelling pattern in the spleen of control animals was generally similar to that described by Harris and Pelc (1970) for short-term exposures. Labelled cells were found in the subcapsular region, around the trabeculae and randomly in the red pulp and occasionally in the mantle layer of the Malpighian follicles. In the spleen of stilboestrol treated animals progressive changes with time were observed and were most marked in the subcapsular region. This region became infiltrated with cells, the majority of which were labelled by Day 3 after treatment. An increase in the number of labelled cells was also seen in the red pulp and the marginal zone while the lymphoid follicles, which in the control animals contained rather few labelled cells, showed little change.

DISCUSSION

Intravascular clearance rates of test materials as a measure of RES phagocytic activity should always be accompanied by organ distribution studies in order to evaluate the relative involvement of the major sites of phagocytosis. This is particularly important when studying enhancement or depression of RES activity since it has been shown recently that various treatments which modify the RES can have differential effects on the different test materials used with respect to their organ localization (Munson, Regelson and Wooles, 1970; Di Luzio and Morrow, 1971). The results of the present series of experiments stress the importance of the same principle when considering the effect of oestrogen induced stimulation of the RES.

The stimulatory effects of various oestrogens on the phagocytic activity of the RES have been extensively studied using histological and intravascular clearance methods (cf. Vernon-Roberts, 1969), but the relative phagocytic activity of the liver and spleen has been seldom appreciated (Heller *et al.*, 1957; Perrin, 1965). As demonstrated by the present results, oestrogens can alter both the qualitative and the quantitative aspects of tissue localization of test materials. These changes were particularly marked in the spleens of stilboestrol treated animals with respect to colloidal carbon and SRBC. Histologically, carbon localization in the spleen showed an altered distribution with almost complete disappearance from the red pulp and accumulation in the marginal zones around the Malpighian follicles. This redistribution appeared to be characteristic of stilboestrol treated animals since it was not found after treatment with endotoxin. The differential effect of these 2 RES stimulants was also evident from uptake studies of colloidal carbon and even more so of ^{51}Cr-labelled SRBC. As opposed to endotoxin, stilboestrol diminished the splenic uptake of colloidal carbon and caused a marked reduction of up to ten-fold of splenic SRBC uptake over a wide range of doses tested. The hepatic uptake was, however, increased after both treatments and in the case of SRBC was particularly evident at an early time after their injection (*i.e.* 15 min) before the vascular clearance was completed.

The reduced splenic uptake of particulate materials in stilboestrol treated mice is of special interest for 2 reasons. First, such an alteration in splenic localization is at variance with the effects of other RES stimulants such as endotoxin, zymosan (Cutler, 1960, and unpublished) and *Corynebacterium parvum* (unpublished). Secondly, in the case of particulate antigens, reduced splenic uptake can be associated with a diminished antibody response (Warr and Šljivić, 1972; Šljivić and Warr, 1973*b*).

There does not seem to be any evidence to suggest a direct depressive effect of stilboestrol on RE cells in the spleen to account for the reduced uptake of test materials. This is, however, difficult to demonstrate conclusively, either *in vitro* because of the lack of adequate methods or *in vivo* because of the presence of the liver and other sites of phagocytosis. In order to test splenic uptake adequately and independently from other sites a test material which is selectively localized in the spleen would be required. Heat-damaged syngeneic cells, which are preferentially but not selectively sequestered by the spleen, partially meet this requirement. With this limitation, the results of the experiment in which heat-damaged MRBC were used indicate that the capacity of the spleen to take up particulate material is not reduced after administration of stilboestrol and that some other explanation must be sought to account for the reduction of uptake of other particles. All other particulate materials for which reduced splenic localization was found, such as colloidal carbon, SRBC (present results and Perrin, 1965) and colloidal chromium phosphate (Heller *et al.*, 1957), have in common that they are readily taken up by the liver. Under conditions of competition between these 2 major sites of phagocytosis, in which the liver has a much greater share in absolute terms, a marked increase in liver activity, such as after administration of stilboestrol, would result in a reduced splenic localization. This conclusion has circumstantial support from a number of observations: (a) the relative splenic uptake of SRBC increases the more the system is saturated by increasing doses of SRBC injected; (b) the characteristic pattern of carbon localization in spleen sections of stilboestrol treated animals can be reproduced by reducing the amount of carbon injected; (c) a dose of colloidal carbon which causes RES " blockade " in control animals and diverts the subsequently injected SRBC from the liver to the spleen (Souhami, 1972) increases the splenic uptake of SRBC in mice stimulated with stilboestrol as compared to that in non-blockaded stimulated animals. Although this postulated mechanism explains the impaired sequestration of SRBC and colloidal carbon in the spleens of stilboestrol treated animals, it is not understood why other agents which stimulate the RES and increase the liver uptake behave differently from stilboestrol with respect to the spleen.

There seems to be little doubt as to the mechanism of the increased rate of blood clearance and liver uptake after administration of stilboestrol. Although increase of serum γ-globulin levels (Nicol *et al.*, 1964) and natural antibodies (Von Haam and Rosenfeld, 1942) have been reported to occur after administration of oestrogens, the results of the serum transfer experiment suggest that an alteration of opsonic factors is not the cause of more efficient clearance and liver uptake of SRBC in mice. This is supported by the finding that stilboestrol altered the organ distribution of SRBC in primed mice which presumably had high opsonin levels. Stilboestrol causes liver and, less regularly, spleen enlargement but the rate of blood flow per unit weight of liver is not increased (Mouton, 1962). As indicated by Mouton (1962) stilboestrol exerts a direct effect upon macrophages in the liver. The present results, showing increased incorporation of [^{3}H]thymidine into the liver, increase in the number of Küpffer cells and a marked increase in the proportion of labelled Küpffer cells after administration of stilboestrol, are in general agreement with the findings of Kelly and co-workers after administration of oestradiol (Kelly *et al.*, 1960, 1962). It has been suggested that the increase in the number of Küpffer cells and increased rate of DNA synthesis reflect a local proliferation of these cells (Kelly and Dobson, 1971) rather than migration and transformation of blood-borne precursor cells (Kinsky *et al.*, 1969).

It is more difficult to interpret increased incorporation of [^{3}H]thymidine into the spleens of stilboestrol treated animals. Labelled cells were found primarily in the subcapsular region and not in the areas which contain most active phagocytic cells, such as the marginal zone. This would suggest that macrophages in the spleen are not activated in the same manner as those in the liver. However, the type of cells which were labelled could not be identified with certainty in spleen sections. It has been reported that administration of oestrogens stimulates erythropoiesis in the mouse spleen and that this is associated with increased radioresistance (Thompson *et al.*, 1969). If the cells which were found to be labelled in the present experiments are primarily erythroid, this would indicate that oestrogens have a selective effect on erythropoiesis in the mouse spleen since similar treatments had no effect on thymidine incorporation into bone marrow cells (Šljivić and Warr, 1973*b*) and reduced the number of stem cells in the bone marrow capable of migrating to form spleen colonies (Thompson *et al.*, 1969).

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