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EXPERIMENTAL OBSERVATIONS ON A TEST FOR SYNOVIAL PERMEABILITY

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The possibility of discovering therapeutic agents which have an action on arthritis like that of cortisone naturally depends on the availability of suitable tests on laboratory animals. Methods which measure synovial permeability, which is presumably an important factor in arthritis, have been known for many years. In one of these methods (Seifter, Baeder, and Begany, 1949) the permeability of the synovial membrane under different experimental conditions was tested by injecting phenol red into the talo-crural articulation, and then measuring its output in the urine. Synovial permeability was increased by hyaluronidase; it was decreased by cortical extracts, steroid compounds, or other compounds which possessed an action against arthritis (Bertolani, Lorenzini, and Bonati, 1951; Bianchi and Meli, 1952; Lorenzini, Bonati, and Bertolani, 1951; Thieblot, Simon, Laforet, and Berthelay, 1951; Seifter *et al.*, 1950). This test, which depends on the intra-articular injection of phenol red, does not involve synovial permeability alone. The appearance of phenol red in the urine also depends on the state of the interstitial tissue, of the capillaries, and of the kidney.

The method used by Coste, Bourel, and Delbarre (1951), which is based on the rate of liquid entering a rabbit's articulation, is independent of the action of the kidneys. However, it still involves not only the synovial membrane but also the tissues and capillaries, whereas for the clinical evaluation of anti-arthritic drugs attention should especially be centred upon their action on the synovial membrane. For this reason, several drugs possessing anti-arthritic activity have been studied by the intra-articular injection test, and by a modification of it devised to identify their exact site of action.

METHODS

Male rabbits of about 2 kg. weight were anaesthetized by intravenous injection of 40 mg./kg. of 5-5' *iso*amylethylbarbituric acid (Ethamyl Zambeletti). They were placed on their backs and a soft rubber catheter was inserted through the urethra into the bladder, which was washed with physiological saline. The catheter was retained there for the duration of the experiment. It was necessary to keep the animal completely anaesthetized and the abdominal muscles perfectly relaxed by administering ether occasionally, in order to avoid expulsion of the catheter. This did not affect kidney function or the elimination of phenol red.

In the first group of experiments 0.25 ml. of a 0.5% (w/v) solution of phenol red in physiological saline was injected into the right talo-crural articulation with an intradermal needle (No. 18). Immediately after the injection the right paw was extended and flexed five times so as to facilitate the spreading of the dye. In order to avoid any disturbances in the general circulation, the right posterior paw was fixed by a noose placed caudal to the articulation.

The bladder was emptied after 1, 2, and 3 hours, and was washed each time with about 90 ml. of physiological saline. Each sample was then made up to 100 ml. As the samples were frequently shown to contain blood, by the benzidine test, they were submitted to deproteinization before measuring their dye content. Then 0.08 ml. of a 45% (w/v) $ZnSO_4$ solution and 0.15 ml. of 1 N-NaOH were added to 10 ml. of liquid. After boiling for 3 minutes the solution was filtered through a sintered glass filter (Jena 3G4) and made up to 20 ml. A few drops of 3 N-NaOH were added and the red colour which developed was read on a Pulfrick spectrophotometer, using filter S.55. The quantity of the dye excreted was calculated from a standard curve.

In a second group of experiments 0.25 ml. of a 0.5% phenol red solution in physiological saline was injected into the periarticular connective tissue. The phenol red was then determined in the urine as previously described.

In a third group of animals the recovery of phenol red in the urine was followed after intravenous injection of 5 ml. of a 0.025% solution of the dye.

Distribution of Phenol Red between Blood and Tissue.—Finally another group of experiments was performed in order to measure the distribution of phenol red between blood and tissue fluid. The rabbits were anaesthetized by intravenous injection of

ethylurethane (1 g./kg.) and a ligature was tied round the renal pedicles in order to eliminate renal excretion. Phenol red 5 mg./kg. was then injected intravenously and the plasma concentration of the dye was measured during the next 2–3 hours. Hence the diffusion rate of phenol red from the blood to the tissues could be estimated, assuming that diffusion across the capillary membrane is a purely physical process. 3 ml. of blood was withdrawn at 5, 35, 65, 125, and 185 minutes after the injection, and was mixed immediately with 0.3 ml. of a 1% sodium oxalate solution in physiological saline. After centrifuging, the plasma was diluted tenfold with distilled water, a few drops of 3 N-NaOH were added, and the intensity of the colour developed was read as usual at 550 mμ. The solution did not need to be deproteinized, as haemoglobin was not usually present; even if it were occasionally present in small amounts, after dilution and alkalinization it does not interfere with measurements at 550 mμ.

If a substance diffuses passively from plasma to the intercellular tissue fluid through the capillary wall, considered as a uniform physical membrane, the concentration of this substance (C) should decrease in the plasma according to the following equation:

$$C = C_o \frac{V_S}{V_t + V_s} + C_o \frac{V_t}{V_t + V_s} e^{-kt(1+V_s/V_t)} \quad (1)$$

where C_o is the concentration at zero time, V_s is the volume of plasma, V_t the volume of the tissue fluid, and K is the coefficient of diffusion of the substance through the capillary wall.

It is possible to transform this equation into a logarithmic one:

$$\ln (C - C_oA) = \ln C_oB - \frac{kt}{B} \quad (2)$$

where $A = \frac{V_S}{V_t + V_s}$, $B = \frac{V_t}{V_t + V_s}$, and $C - C_oA$ is the difference between C at a time and C after complete equilibrium.

From each experiment plasma concentrations are obtained 5, 35, 65, 125, and 185 minutes after the injection; the course of the plasma concentration curve obtained is reproduced in Fig. 1. If the logarithms of the difference between the concentrations after 3, 35, and 65 minutes and that at 125 minutes are plotted on the ordinate and the corresponding times on the abscissa, theoretically a straight line should be obtained (Fig. 2). As shown in Fig. 1, the plasma concentration of dye at 125 minutes after injection represents nearly an equilibrium value.

If B is constant, the slope of this line, represented by K/B, is related to the diffusion coefficient of phenol red through the capillaries, and, if K is constant, the slope of this line is related to the ratio between tissue fluid volume and plasma-plus-tissue-fluid volume. K/B was calculated statistically for each experiment and for each group of animals.

The distribution of phenol red between blood and tissue fluid is presumably more complex than we have assumed; nevertheless this simple procedure does permit us to estimate differences in the distribution rate between treated and control animals.

All the animals except the controls were previously treated for four days with the drugs under experiment

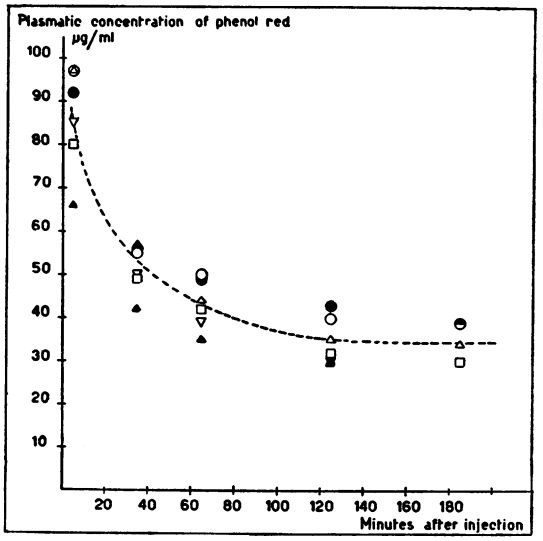

FIG. 1.—Rabbits with tied renal pedicles. Plasma concentration of phenol red plotted against time in minutes after intravenous injection of 5 mg./kg. of the dye. The different types of symbols designate plasma concentration of dye in different animals.

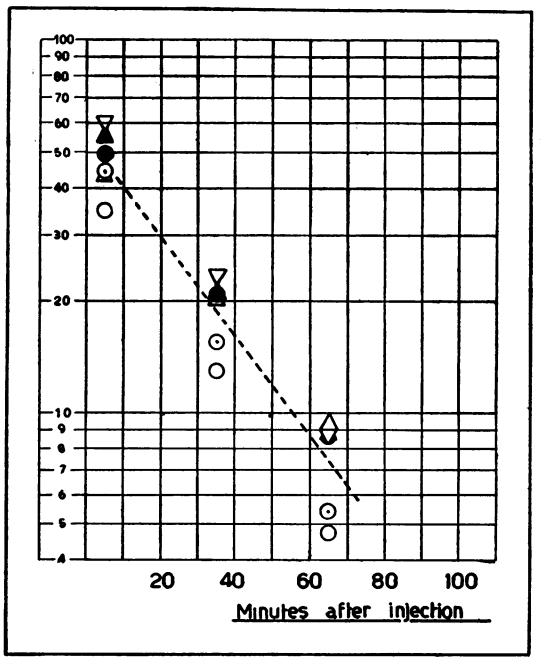

FIG. 2.—Rabbits with tied renal pedicles. Differences between plasma concentration of phenol red at 5, 35, 65 minutes and concentration at 125 minutes after intravenous injection of 5 mg./kg. of the dye plotted against time in minutes. The different types of symbols designate results in different animals.

cortisone acetate, pregnenolone, 21-acetoxypregnenolone, desoxycorticosterone acetate (DOCA), benzoylcarbinol acetate (Logemann and Giraldi, 1951; structural formula: C_6H_5-CO-CH_2-O-$COCH_3$), and sodium salicylate. These products were injected intraperitoneally, 300 mg./kg. in all (75 mg./kg. per day). DOCA was injected intraperitoneally, 20 mg./kg. in all (5 mg./kg. per day). A commercially available suspension of cortisone (Cortone Merck) was used. All the other products were suspended at a 2% (w/v) concentration in a 10% (w/v) gum acacia solution.

The Intra-articular Injection of Phenol Red.—Some observations must be made about the technique described by Seifter *et al.* (1949) for intra-articular injection of phenol red. The noose fixing the paw must always be put caudal to the articulation when the injection is made, because a slight pressure on the upper part retards absorption. When a pressure of 50 cm. H_2O was exerted by a sphygmomanometer cuff placed cranial to the articulation, the phenol red absorbed was 37% less in one hour and 22% less in three hours than what it was with the articulation not constricted (mean values of four experiments).

The temperature must be maintained constant throughout experiments, as the circulation also varies with the external temperature; the phenol red absorbed by the articulation of animals maintained at a constant temperature of 17° C. throughout an experiment was 39% less in one hour and 22% less in three hours than what it was in animals maintained at a temperature of 21° C. (mean values of four experiments).

Animals cannot be used more than once. When the same animal was used ten days after a first experiment, the absorption of phenol red was found to be 22% less in one hour and 28% less in three hours (mean values of four experiments) than it had been in the first experiment. In the second test the injection was made into the articulation not previously used. These precautions were always observed in all tests.

Seifter, Baeder, and Blumenthal (1952) have also described the factors influencing synovial membrane permeability.

Results

The compounds studied have been divided into three groups, according to their type of action (Table I).

Group 1 (Cortisone, Na salicylate, benzoylcarbinolacetate)

These three compounds act on the synovial membrane only. Whereas absorption of phenol red from the articular cavity was partially inhibited (this result is statistically uncertain with cortisone) absorption after subcutaneous injection was not altered, nor was passage of phenol red through the capillaries or kidney altered.

Group 2 (Desoxycorticosterone-acetate (DOCA))

This compound acts on subcutaneous tissue, but not on the kidney, capillaries, or synovial membrane. The amount of phenol red eliminated after intra-articular injection of dye was nearly equal to that obtained after injection into the subcutaneous tissue near to the articulation.

Group 3 (Pregnenolone and 21-acetoxypregnenolone)

The strong inhibitory action of these products on the elimination of phenol red was due wholly or partially to a mechanism different from the previous one. When phenol red was injected into the blood stream less was eliminated than in the control observation. This could be explained by the inhibition of phenol red elimination through the kidneys.

For pregnenolone there is also an increase of the value K/B for equation (2). This could be explained by an increase in capillary permeability or bv a decrease in absorption capacity of tissues for phenol red (V_t).

TABLE I

RENAL EXCRETION OF PHENOL RED 1 HOUR AND 3 HOURS AFTER INTRA-ARTICULAR, PERIARTICULAR, OR INTRAVENOUS INJECTION (PERCENTAGE OF INJECTED AMOUNT)

For K/B see text. Probable deviations are quoted after each figure*

i.p. Injection of †	Intra-articular		Periarticular		Intravenous		Capillary Permeabi ity K/B 2·3
	1 hr.	3 hr.	1 hr.	3 hr.	1 hr.	3 hr.	
O (controls)	54·2± 4·4	76·8± 5·0	55·4±10·4	88·0±10·5	72·5± 7·9	86·8± 7·3	−0·0134±0·0009
Cortisone acetate ..	48·0±18·0	72·5±14·8	52·2±10·3	79·8± 6·1	—	—	
Na salicylate	39·1± 9·5	62·7± 6·2	58·4± 6·9	85·2± 2·8	—	—	−0·0149±0·0029
Benzoylcarbinolacetate ..	38·2± 6·4	62·2± 3·9	58·1± 6·3	84·7± 4·9	—	—	−0·0154±0·0033
Desoxycorticosterone acetate	46·0± 6·4	69·4± 7·0	46·9± 5·7	82·4± 2·0	74·9± 2·9	88·8± 2·8	−0·0157±0·0019
21-Acetoxypregneno'one	20·4± 7·1	46·9± 4·2	19·1±11·7	45·0±13·6	58·4± 8·6	77·6± 7·4	−0·0139±0·0026
Pregneno'one	28·1± 7·5	60·6± 6·2	29·7± 8·1	64·8±12·5	33·1±14·5	52·9±13·3	−0·0208±0·004

* These figures are based on from 4–6 experiments for each compound, and from 6–16 experiments on the control observations.
† For amount injected, see text.

Discussion

From our experiments it appears that the decreased urinary elimination of phenol red after intra-articular injection into rabbits cannot always be explained in the same way; the experimental results may have an articular explanation or an extra-articular one (subcutaneous, renal, or circulatory).

Sodium salicylate, which has a certain anti-arthritic action, shows also a clear action on synovial permeability; on the contrary, cortisone, which is notoriously an anti-arthritic compound, shows a less evident effect on the synovial membrane. Cortisone also failed to cause any significant alteration of synovial permeability, according to Paul, Hodges, Knouse, and Wright (1952) and Hidalgo, McClure, Henderson, Whitehead, and Smyth (1952). Benzoylcarbinolacetate also had a clear action on the synovial membrane, but its therapeutic effects are uncertain. Another compound, cinchophen, used successfully in therapy, had a strong inhibitory action on renal elimination of the dye and cannot therefore be used for this test (Bianchi and Bernini, 1952). Of the other compounds investigated, none had a specific action only on the synovial membrane; this suggests that a different mechanism of action exists. The greater activity of 21-acetoxypregnenolone compared to cortisone, which was described by Seifter *et al.* (1950) and confirmed by others (Bianchi and Meli, 1952), had not the value attributed to it, as the action of the two compounds was different. In opposition to other opinions DOCA had no specific action on the permeability of the synovial membrane.

It is difficult to compare our results with those obtained with other tests based upon diffusion phenomena, such as those involving the Indian-ink intradermal diffusion or the passage of water through connective tissue membranes (Bianchi and Meli, 1952; Capraro and Meli, 1951). These tests are based on the inhibitory action of compounds on diffusion increased by hyaluronidase. The elimination of phenol red after injection into the articular cavity, as well as the antihyaluronidase activity, is influenced by other properties of compounds which modify the permeation phenomenon as shown for pregnenolone and 21-acetoxypregnenolone. For this reason, according to our opinion, a screening test for synovial permeability must not be limited to the single measurement of the excretion of phenol red injected into the articular cavity. Obviously, if the elimination of phenol red were not modified, it would be unnecessary to look further. But if elimination is delayed, then it is necessary to examine the various possible mechanisms.

Summary

1. The renal excretion of phenol red after intra-articular injection in the rabbit treated with different compounds does not depend only upon the permeability of the synovial membrane. The permeability of the connective periarticular tissue, of the capillaries, and of the renal tubules may also modify the renal excretion.

2. By using various techniques (intra-articular injection of phenol red; injection of phenol red into the periarticular connective tissue; intravenous injection of phenol red in normal rabbits; intravenous injection of phenol red in rabbits with a ligature tied round the renal pedicles) it is possible to identify the site of action of the compounds under investigation.

3. Sodium salicylate and benzoylcarbinolacetate diminished the permeability of the synovial membrane. Cortisone had an uncertain action.

4. Desoxycorticosterone acetate diminished the permeability of subcutaneous periarticular tissues.

5. Pregnenolone and 21-acetoxypregnenolone mainly decreased the renal excretion of phenol red.

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