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STUDIES ON THE BLOOD-BRAIN BARRIER. II. ATTEMPTS TO INFLUENCE THE PASSAGE OF SUBSTANCES INTO THE BRAIN

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If by exhibition of a drug or by other means it were possible temporarily to modify the permeability of the blood-brain barrier, the experimentalist would acquire a technique of no little value for investigating some outstanding problems of neurophysiology and of the pathogenesis of nervous disease. The literature contains a number of references to measures which are supposed to increase or to decrease the permeability of the blood-brain or the blood-cerebrospinal fluid barrier. The evidence on which the claims are based, however, is not always unequivocal, nor is it always certain that the effects observed are due wholly or in part to altered permeability *per se* (see Broman, 1941). In other work no specific claim of altered permeability is made, though often this interpretation of the effects described is implicit. In the present investigation we sought by rather drastic measures to modify the passage into the brain first of dyes, then of convulsant drugs and morphine, and later of substances the presence of which in the brain can be detected by chemical means; the reasons for the choice of many of the measures are apparent from the brief résumé of the literature given below.

Measures reported to influence the passage of substances into the brain or cerebrospinal fluid

Adrenaline.—Stern, Slatowierow, and Kremlew (1927) showed that in cats and rabbits the administration of adrenaline caused trypan blue to colour the brain, which normally it fails to do.

Friedemann and Elkeles (1932a and b) and Friedemann (1937) described an "auxo-neurotropic" effect of adrenaline and pituitrin (but not of insulin or thyroxin). Combining adrenaline with pituitrin avoided the undesirable side-effects of adrenaline given alone. Injected intravenously into rabbits these substances increased the permeability of the blood-brain barrier for narcotics, convulsants, and dyes which normally penetrate the brain, but not for drugs, dyes, and toxins to which the barrier is normally impermeable. The tolerated dosage of adrenaline and pituitrin varies greatly in different species of animals (see Hurst, 1944, p. 114).

Theocin.—Fröhlich and Zak (1927) and Franceschetti and Wieland (1928) found that theophyllin sodium acetate (theocin) injected intravenously favoured the passage of drugs into the brain or cerebrospinal fluid. The former obtained positive results with acid dyes and morphine in frogs, morphine in rabbits and cats, magnesium chloride in rabbits, and potassium ferrocyanide in mice and guinea-pigs. Inconstant results were obtained with bromide, and negative results with antipyrin in mice and

guinea-pigs. The latter authors similarly increased the passage of an arsenical into the cerebrospinal fluid of rabbits.

Urethane.—Although no observations were made on the blood-brain barrier, Hurst (1942) noticed that monkeys receiving urethane developed severe and generalized oedema suggestive of an effect on capillary permeability, an effect already demonstrated in perfused preparations by Landis (1927).

Histamine.—Sprockhoff (1935) reported that subcutaneously administered histamine increased greatly the passage into the cerebrospinal fluid of acid fuchsin given intravenously to dogs.

Hexamine.—Le Fèvre de Arric and Millet (1926) claimed that urotropine (hexamine) given intravenously before herpes virus enabled the latter to pass the blood-brain barrier and set up encephalitis in rabbits. From the facts now known regarding the pathogenesis of herpetic infection of the nervous system after intravenous inoculation (Cooke, Hurst, and Swan, 1942) it is not certain that this claim is valid.

Stern and Zeitlin (1927) and Stern, Kassil, and Lokschina (1927) found that urotropine in cats and rabbits favoured the passage through the barrier of trypan blue, but not of congo red, sodium ferrocyanide, sodium iodide, or bismuth subnitrate. Mutermilch (1926) forced antibodies and an arsenical through the blood-cerebrospinal fluid barrier, and Stern, Kassil, Lokschina, Romel, and Zeitlin (1928) haemolysins through this barrier by the same treatment.

Glycerol, hypertonic and hypotonic solutions.—King (1942) showed that *sudden* dehydration, resulting from intraperitoneal or intramuscular administration of 50 per cent glycerol or of hypertonic (30 per cent) saline, greatly facilitated invasion of the brains of mice by the virus of equine encephalomyelitis given intramuscularly (but not intranasally or intraocularly). The procedures mentioned lowered the weight of the brain and increased the percentage of solids in it.* No facilitation followed violent shifts of electrolytes by the administration of large doses of distilled water or of 5 per cent glucose. The slow dehydration consequent upon deprivation of water for 24–48 hours had no facilitating effect.

Previously, Stern, Zeitlin, and Gozman (1928) had found that when the osmotic pressure of the blood of cats and rabbits exceeded $\triangle = 0.72$ haemolysins passed into the cerebrospinal fluid; above $\triangle = 0.8$ sodium iodide and potassium ferrocyanide passed, and at $\triangle = 0.9$ trypan blue and congo red. Similar effects followed falls of osmotic pressure below $\triangle = 0.45$. The altered osmotic pressures were brought about by intravenous hypertonic Ringer solution, concentrated glucose solution, or strongly hypotonic solutions.

Insulin and coal-gas.—Insulin shock and poisoning with coal-gas enabled Findlay (1942) to localize in the brains of adult mice neurotropic yellow fever virus injected intraperitoneally; normally it is only in very young mice that the virus produces encephalomyelitis after inoculation by this route (Theiler, 1930). Findlay suggested that capillary walls damaged by lack of oxygen during the hypoglycaemic crisis or otherwise may permit virus to seep through.

Siengalewicz (1924) exposed rabbits to coal-gas and modified the permeability of the cerebral blood vessels so that they readily passed trypan blue.

* At autopsy on mice treated with glycerol we have noticed through the thin cranial vault that the brain is of a deep plum-colour, and often that free blood lies in the subdural space. Histologically, in specimens which have been fixed before the cranial vault has been removed, not only are the cerebral vessels distended with closely packed red-blood-corpuscles, but masses of these may lie free outside the vessels of the meninges. These observations point to a considerable degree of disturbance of the cerebral circulation.

Stern and Lokschina (1927) and Stern, Kassil, and Lokschina (1927) observed that exposure to carbon monoxide assisted the passage of bismuth subnitrate and of colloids such as trypan blue and congo red into the brain and cerebrospinal fluid of rabbits and mice, but did not have this effect with sodium ferrocyanide and sodium iodide. From this and other work they concluded that altered permeability for one substance does not allow prediction of the effect with other substances more or less similar chemically or physico-chemically.

Ether.—Teague and Perdue (1948) reported that the cerebrospinal fluid of dogs anaesthetized with ether contained 80 per cent more sulphathiazole (administered intraperitoneally) than did that of unanaesthetized animals. Anaesthesia with dial-urethane led only to a 20 per cent increase, while chloroform anaesthesia had no such effect. The authors relate variations in the passage of substances from blood to cerebrospinal fluid to (*a*) the total blood-flow through the brain and (*b*) the state of permeability of the cerebral capillaries.

Vital dyes.—Cobb, Cohen, and Ney (1938) observed that intraperitoneally administered brilliant vital red and neutral red increased the resistance of mice and rabbits to various convulsant drugs (cocaine, strychnine, picrotoxin, etc.) but not to electrically induced convulsions. Aird (1939) and Aird and Strait (1944) confirmed this observation and suggested that the cause is a decreased permeability of the blood-brain barrier, since spectrophotometric determinations showed a reduction by 30–40 per cent in the amount of cocaine passing into the brain and cerebrospinal fluid of dogs and cats treated with brilliant vital red or trypan red. Aird and Strait found that the maximum effect with trypan red, a dye closely related to brilliant vital red, occurred after three or more daily doses of the dyestuff.

METHODS

A previous article (Davies and Hurst, 1948) outlined the precautions taken in selecting and preparing mice (20 g. ± 0.5 g.) for experiment.

In the experiments with dyes we gave groups each of six mice two intraperitoneal doses at an interval of one hour; the inoculum measured 0.25 or 0.5 c.c. and contained the largest amount of dye tolerated in earlier experiments in which about 100 dyes were examined for ability to stain the nervous tissue. Fifteen minutes after the second dose we administered the substance under test for an action on the blood-brain barrier; for brevity, we shall refer to these substances as "adjuvant" drugs. The following "adjuvants," dissolved in 0.1 c.c. water, were injected intravenously in exactly 10 seconds:—Adrenaline, 0.005 mg., and pituitrin, 0.1 unit; theocin, 4 mg.; sodium lactate, 16 mg.; urethane, 20 mg.; histamine, 7 mg.; hexamine, 50 mg.; insulin, 0.6 units. Mice receiving insulin had been fasted during the previous 24 hours. Glycerol (50 per cent v/v) and hypertonic saline (30 per cent w/v) were given intramuscularly in doses of 0.25 c.c. Control mice always received an appropriate dose of water. In other experiments, starting 15 minutes after the second injection of dye we maintained anaesthesia with ether for 30 minutes, or exposed the mice intermittently to coal-gas to produce unconsciousness for a total of 10 minutes. In these experiments the controls remained without treatment. An hour after administration of the "adjuvant" drug, or after withdrawal of the anaesthetic or coal-gas, the mice were killed. After being inspected, their brains were fixed overnight in a minimal quantity of formol-saline, after which a cut surface was compared with water-colour washes matching the tints obtained in preliminary experiments with various doses of dye. To each arbitrarily chosen depth of colour we assigned a number, and calculated the total "score" for each group of mice. When any suggestion of an effect was obtained, we repeated the test four or six times.

In a second series of experiments, many of the drugs mentioned above were tested for an effect on the mortalities resulting in mice from the administration of the convulsant drugs studied in our previous work (Davies and Hurst, 1948)—strychnine, cocaine, and picrotoxin. In most instances we injected the convulsant, dissolved in 0.1 c.c. water, intravenously in exactly 10 seconds, and followed it immediately by the "adjuvant" drug similarly dissolved. Glycerol (50 per cent v/v) was given intramuscularly in a dose of 0.25 c.c., and the convulsant drug intravenously 10 minutes later. One set of controls received convulsant followed by the appropriate dose of water, another set the "adjuvant" drug followed by water. A single experiment consisted in testing a given "adjuvant" drug against all three convulsants, using groups each of 10 mice; the observations were repeated on other days, usually by a different operator.

Previous workers using these convulsant drugs have observed the intensity of the ensuing convulsions. Where a large number of mice are under test at one time, we felt that the observer might fail to notice a slight convulsive tendency in a particular animal, and that error was less likely if mortality was taken as the index of action of the drug. We realize that, while with strychnine and picrotoxin death is due purely to the action of the drug on the central nervous system, with cocaine other actions may be partly responsible.

We carried out tests with morphine on rats of 150–200 g., noting their reaction-time to a thermal stimulus according to the method of Davies, Raventós, and Walpole (1946). After a preliminary test to ascertain that their responses were within normal limits, the animals were arranged in groups of five with particular reaction-times evenly distributed throughout. Not less than 48 hours after the preliminary test, the observations with morphine and "adjuvant" drugs were made in a manner comparable with that described for the convulsant drugs.

In a further series of experiments, sulphanilamide or sulphanilic acid dissolved in 0.1 c.c. water was given intravenously to mice. Ten minutes later the "adjuvant" drug or water was administered by the same route (glycerol was given intramuscularly immediately after sulphanilamide or sulphanilic acid), and ten minutes later (twenty minutes in the case of glycerol) the animals were killed. The time of autopsy relative to that of administering sulphanilamide was determined from experiments to be reported in a future publication. Chemical estimations followed the method of Rose and Bevan (1944). A single experiment included one or more mice on one or more dose-levels or sulphanilamide or sulphanilic acid for each "adjuvant" under test, together with two or more control mice receiving water instead of "adjuvant." The observations were repeated once or more as necessary. In the experiments with coal-gas, the mice were kept unconscious for a total of 10 minutes immediately before and again immediately after injection of the sulphanilamide. In the experiments with ether they were kept continuously anaesthetized from the time of giving sulphanilamide and were killed after 30 or 20 minutes (sulphanilamide and sulphanilic acid respectively).

Finally we examined trypan red (C.I.438), brilliant vital red (C.I.456), and neutral red (C.I.825) for a restraining effect on the passage of drugs into the brain. With the first two we gave intravenously three daily doses of 0.1 c.c. of a 0.5 per cent (w/v) solution (1 c.c./100 g. of 2 per cent (w/v) solution intraperitoneally to rats), and on the fourth day the convulsant drug, morphine, or sulphanilamide. Because neutral red (0.1 c.c. of 0.2 per cent (w/v) intravenously to mice; 1 c.c./100 g. of 1 per cent (w/v) intraperitoneally to rats) is removed much more easily from the tissues, we administered a further dose of the dye half an hour before the convulsant drug, morphine, or sulphanilamide on the fourth day. Control animals received similar preliminary treatment with water. For the chemical estimations a second series of

controls received dye followed by water instead of sulphanilamide, as a check on the influence, if any, of dye extracted from the capillary endothelium or brain on the reading of the colour-reaction engendered in the test. Neither the dye nor the products of its breakdown had any appreciable effect.

RESULTS

Experiments with dyes

The dyes used were as follows. *Acid dyes:* azogeranine, C.I.31 ; benzopurpurine, C.I.448 ; brilliant vital red, C.I.456 ; chlorazol fast scarlet, C.I.327 ; chromazol yellow, C.I.441 ; dianil blue, C.I. 465 ; pontamine sky blue, C.I.518 ; new wool blue ; wool blue. *Triphenylmethanes:* disulphine blue VS ; isamine blue, C.I.710. *Basic dyes:* bismarck brown G, C.I.331 ; methylene blue, C.I.922 ; neutral-red chloride, C.I.825. These dyes represent a number of chemical types. They were chosen on account of their relatively low toxicities from about a hundred dyes which had previously been tested for ability to pass the blood-brain barrier. The acid dyes normally pass the barrier with difficulty, if at all, when administered in the largest doses tolerated. Microscopically, in slightly coloured brains, particles of dye may sometimes be seen in the capillary endothelium, in mesodermal cells closely applied to the outer walls of the capillaries, or in both ; if any dye penetrates the nervous tissue proper it is present in too low a concentration to be seen in frozen sections cut at 25 μ. On the other hand, many of the triphenylmethanes, which though classed as acid dyes behave in fact as "*zwitterions*," colour the brain quite well ; this is so with disulphine blue, whereas isamine blue behaves like acid dyes generally. The wool blues (anthraquinones) also stain the brain readily. As is well known, most basic dyes enter the brain freely.

We studied the effect of adrenaline and pituitrin, theocin, sodium lactate, urethane, histamine, hexamine, glycerol or hypertonic saline, insulin-shock, ether-anaesthesia, and exposure to coal-gas upon the passage into the brain of these dyes. The full range of dyes was not tested with every "adjuvant" drug or treatment, but on every occasion several acid dyes, the triphenylmethanes, and at least two basic dyes were included.

In most tests no distinct difference existed between mice receiving dye succeeded by water and those receiving dye succeeded by a drug or other treatment. In the following instances moderately increased staining appeared to result ; adrenaline and pituitrin with bismarck brown, neutral red, and methylene blue ; theocin with bismarck brown ; histamine with neutral red ; ether-anaesthesia with bismarck brown, neutral red, methylene blue, and new wool blue ; exposure to coal-gas with bismarck brown, neutral red, methylene blue, and disulphine blue. It will be noted that all these dyes normally pass the blood-brain barrier without difficulty. The only outstandingly positive results were with glycerol or with hypertonic saline. Here, in repeated tests, there occurred slightly increased coloration with such acid dyes as chlorazol fast scarlet and brilliant vital red, which normally penetrate the brain with great difficulty. The coloration with pontamine sky blue and disulphine blue was markedly deepened, as was also that with bismarck brown, neutral red, and methylene blue. Urethane appeared to diminish penetration by neutral red and methylene blue.

On the whole, for a variety of reasons, we did not consider these experiments with dyes completely satisfactory. With any given dose of dye, considerable varia-

TABLE I

MORTALITIES IN GROUPS OF 10 MICE INJECTED WITH A CONVULSANT AND AN "ADJUVANT" DRUG

Glycerol (50 per cent (v/v)) was given in a dose of 0.25 c.c. intramuscularly; all other drugs in a volume of 0.1 c.c. water intravenously. Glycerol was given ten minutes before convulsant; all other "adjuvants" immediately after convulsant.

Convulsant drug	"Adjuvant" drugs													
	Adrenaline 0.005 mg. plus pituitrin 0.1 unit	Water	Theocin 4 mg.	Water	Sodium lactate 16 mg.	Water	Urethane 20 mg.	Water	Hist-amine 7 mg.	Water	Hex-amine 50 mg.	Water	Glycerol 50 per cent (v/v)	Water
Strychnine hydrochloride 0.0107 mg. ..	6, 7	2, 2	5, 7	2, 3	2, 4, 3, 4	2, 4, 6, 7	1, 0, 0, 1	1, 2, 6, 7	7, 4, 9	5, 4, 5	7, 2, 2, 5	7, 4, 4, 5	7, 4, 5, 4	6, 1, 1, 1
Cocaine hydrochloride 0.47 mg. ..	4, 5, 10	3, 3, 7	9, 7	3, 2	4, 6, 9	2, 3, 7	8, 6, 10	3, 4, 7	9, 6	3, 2	5, 6, 3, 6	7, 7, 7, 7	3, 2, 4, 0, 3	2, 3, 7, 4, 8
Picrotoxin 0.066 mg. ..	3, 3 7, 6, 9	0, 1 5, 3, 4	10, 10	3, 1	7, 3 8	2, 1 6	0, 0 1, 0, 0	3, 1 5, 3, 4	7, 4	1, 1	2, 2, 2, 6	4, 4, 4, 9	6, 10, 10, 7	5, 5, 4, 2
0.075 mg. ..														
Water 0.1 c.c.	1, 1, 1, 2, 0		1, 0		0, 0, 0, 0		0, 0, 1, 1, 0		0, 0, 0		0, 0, 0, 0		0, 0, 0, 0, 0	

TABLE II

MORTALITIES IN GROUPS OF 10 MICE INJECTED WITH A CONVULSANT AFTER PRELIMINARY TREATMENT WITH A VITAL DYE

0.1 c.c. of 0.5 per cent trypan red or brilliant vital red was given intravenously on three successive days and the convulsant dissolved in 0.1 c.c. water twenty-four hours after the last dose; 0.1 c.c. of 0.2 per cent neutral-red chloride was given intravenously on four successive days and the convulsant half an hour after the last dose.

Convulsant drug	Trypan red	Water	Brilliant vital red	Water	Neutral-red chloride	Water
Strychnine hydrochloride 0.0107 mg.	5, 4, 5	8, 7, 7	6, 9, 7, 6, 8	5, 4, 5, 7, 6	5, 4, 8, 6, 6	7, 5, 6, 7, 7
Cocaine hydrochloride 0.47 mg.	2, 2, 3	4, 3, 3	8, 4, 6, 6, 8	6, 6, 6, 3, 8	9, 4, 6, 6, 6	7, 3, 8, 7, 6
Picrotoxin 0.075 mg. ..	5, 5, 5	7, 5, 6	4, 2, 3, 4, 5	7, 8, 4, 6, 9	2, 2, 4, 5	3, 5, 5, 7
Water 0.1 c.c.	0, 0, 0		0, 0, 0, 0, 0		0, 0, 0, 0, 0	

TABLE III

MEAN ALTERATION IN SECONDS OF REACTION-TIMES TO A THERMAL STIMULUS IN GROUPS OF 5 RATS INJECTED WITH MORPHINE AND AN " ADJUVANT " DRUG

Glycerol was given intramuscularly; the other " adjuvant " drugs in a volume of 0.25 c.c. intravenously. Glycerol was given ten minutes before morphine; all other " adjuvants " immediately after morphine. The figures show the difference between the mean reaction-times 15 min. after morphine and those before morphine. The figures in parentheses are the grand means for the various groups.

" Adjuvant " drug and dose/100 g.	" Adjuvant " drug and water	Morphine 0.2 mg./ 100 g. and water	Morphine 0.2 mg./ 100 g. and " adjuvant " drug
Adrenaline 0.002 mg. and pituitrin 0.04 unit	0 −0.08 +0.09 (0.10) +0.37	+1.38 +1.48 +1.26 (1.35) +1.26	+2.66 +1.51 +1.40 (2.14) +2.98
Theocin 20 mg.	−0.28 −0.05 −0.19 (−0.24) −0.44	+1.34 +2.00 +1.81 (1.77) +1.94	+1.00 +0.80 +0.38 (0.73) +0.75
Sodium lactate 40 mg. ..	+0.23 +0.52 (0.32) +0.20	+1.76 +1.66 (1.85) +2.14	+1.30 +1.44 (1.89) +2.92
Histamine 17.5 mg. ..	+1.52 +1.10 (1.31)	+1.68 +1.52 (1.60)	+4.16 +4.30 (4.23)
Hexamine 250 mg. ..	−0.04 −0.06 (−0.05)	+1.28 +1.44 (1.36)	+1.12 +1.46 (1.29)
50 per cent (v/v) glycerol 1 c.c.	−0.01 +0.13 (0.06)	+1.46 +1.16 (1.31)	+1.22 +1.05 (1.14)

tions in the depth of staining occurred from mouse to mouse ; moreover, day-to-day variations in repeat experiments seemed difficult to eliminate, while the possibility of subjective errors in reading the results was not absent.

Experiments with convulsant drugs and with morphine

The convulsant drugs studied were those employed in a previous investigation (Davies and Hurst, 1948). Table I shows the mortalities resulting in groups each of 10 mice from (*a*) the convulsant combined with the " adjuvant " drug, (*b*) the convulsant combined with water, and (*c*) the " adjuvant " drug combined with water. Table II presents similar results for the convulsant drugs and vital dyes. Tables III and IV record the observations in groups each of five rats in which morphine was substituted for a convulsant drug. Table V summarizes the conclusions to be reached from these results.

It will be seen that some " adjuvant " drugs, namely adrenaline and pituitrin, theocin and (save for a single observation with strychnine) histamine, consistently increased the mortality due to each convulsant. With many " adjuvants " the overall effect was significant ; had the experiments been repeated a greater number of times it is probable that significance would have been attained with them all, since there were no observations in the reverse direction. On the other hand, adrenaline and pituitrin only slightly increased the effect of morphine, theocin decreased it, and histamine, while markedly increasing the effect, itself altered considerably the time of reaction to a thermal stimulus. The remaining " adjuvants " were even less

TABLE IV

MEAN ALTERATION IN SECONDS OF REACTION-TIMES TO A THERMAL STIMULUS IN GROUPS OF 5 RATS INJECTED WITH MORPHINE AFTER PRELIMINARY TREATMENT WITH A VITAL DYE

1 c.c./100 g. of 2 per cent trypan red or brilliant vital red was given intraperitoneally on three successive days and the morphine dissolved in 0.25 c.c. water twenty-four hours after the last dose. 1 c.c./100 g. of 1 per cent neutral-red chloride was given intraperitoneally on four successive days and the morphine half an hour after the last dose. The first column of figures shows the difference between the mean reaction-times after and before treatment with dye. The second column shows the difference between the mean reaction-times 15 min. after morphine and those before morphine. The figures in parentheses are the grand means for the various groups.

Preliminary treatment	No morphine	Morphine 0.2 mg./100 g.
Water		+1.44 +1.28 (1.29) +1.44 +1.00
Trypan red	+0.09 +0.96 −0.06 (−0.02) −1.08	+1.58 +1.28 (1.23) +1.20 +0.86
Brilliant vital red	−0.04 +0.40 (0.06) −0.18	+0.85 +1.26 (0.96) +0.76
Neutral-red chloride	−0.26 +0.74 (0.15) −0.02	+6.32 +4.76 (5.88) +6.56

TABLE V

EFFECT OF " ADJUVANT " DRUGS AND VITAL DYES ON THE TOXICITY OF CONVULSANTS AND OF MORPHINE. SUMMARY OF CONCLUSIONS REACHED FROM THE DATA IN TABLES I–IV

The level of significance used is P = 5 per cent.

" Adjuvant " drug or vital dye	Effect on the toxicity of			
	Strychnine	Cocaine	Picrotoxin	Morphine
Adrenaline and pituitrin	Significant increase	Increase — nearly significant	Significant increase	Slight increase — not significant
Theocin ..	Significant increase	Significant increase	Significant increase	Significant decrease
Sodium lactate	Decrease — nearly significant in later tests	Significant increase	Significant increase	No apparent effect
Urethane ..	Significant decrease	Significant increase	Significant decrease	Not examined
Histamine ..	Increase — nearly significant	Significant increase	Significant increase	Significant increase
Hexamine ..	Slight decrease— not significant	Decrease — very nearly significant	Significant decrease	No apparent effect
Glycerol ..	Significant increase	Significant decrease	Significant increase	Slight decrease— not significant
Trypan red ..	Significant decrease	Decrease — not significant	Slight decrease— not significant	No apparent effect
Brilliant vital red	Increase — just significant	Slight increase— not significant	Significant decrease	Decrease — nearly significant
Neutral-red chloride	Slight decrease — not significant	No apparent effect	Decrease — almost significant	Significant increase

consistent in their behaviour. Sodium lactate increased mortality from cocaine and picrotoxin and possibly from strychnine, but had no clear effect with morphine. Urethane also differed in its action with the various convulsants. Perhaps the most unexpected result was the decreased effects of cocaine and morphine in the presence of glycerol, since all other work (see above and below) pointed to glycerol as having the most pronounced effect of any " adjuvant " in forcing substances into the brain.

As expected from reports in the literature, trypan red reduced mortality with all convulsants, but with morphine it had no effect. Brilliant vital red and neutral-red chloride also acted very differently with the various test-substances.

The fact that most " adjuvants " and vital dyes produce quite different effects with different test-substances suggests that, without further evidence, we cannot assume these effects to be due necessarily to an increased or decreased passage of test-substance into the brain ; in some combinations, of which perhaps that of morphine and theocin is an obvious example, the final result may be the outcome of synergistic or antagonistic interaction of the two drugs, quite independent of factors of permeability. It seemed that a conclusion could be reached only by chemical estimation of drugs passing into the nervous tissue. For strychnine, picro-

TABLE VI

EFFECT OF "ADJUVANT" DRUGS ON THE CONCENTRATIONS OF SULPHANILAMIDE AND SULPHANILIC ACID IN THE BRAINS OF MICE

Experiments with sulphanilamide*	Dose of "sulpha" drug mg.	Amounts are given in mg. per 100 g. brain. Each represents an observation on a single animal						
		Water (controls)	Adrenaline 0.005 mg. plus pituitrin 0.1 unit	Theocin 4 mg.	Sodium lactate 16 mg.	Urethane 20 mg.	Histamine 7 mg.	Glycerol
1	1.5 3.0	2.6, 4.7 6.6, 9.0	3.7 7.0	4.7 10.5	4.1 8.0	3.9 7.5	5.0 6.9	3.6 9.7
Ratios to geometric mean of controls	1.5 3.0		1.05 0.91	1.33 1.37	1.16 1.04	1.10 0.97	1.42 0.89	1.03 1.27
Geometric mean of ratios			0.98	1.35	1.10	1.03	1.13	1.14
2	1.5 3.0	4.3, 4.9 7.7, 6.8	6.7, 7.5	3.0, 4.1	4.3, 4.0	3.5, 4.3	6.5, 9.2	7.8, 8.0
Ratios to geometric mean of controls			0.79	0.76	0.90	0.85	1.07	1.09
3	2.0	4.3, 3.9, 5.3	4.1, 4.2, 4.0				4.2, 3.9, 4.5	5.3, 5.0, 5.6
Ratios to geometric mean of controls			0.92				0.94	1.17
Grand geometric mean of ratios ..			0.90	1.06	1.01	0.95	1.03	1.14
95 per cent limits			0.77–1.05	0.87–1.29	0.83–1.23	0.78–1.16	0.88–1.21	0.97–1.33
Experiments with sulphanilic acid*								
1	1.5 3.0	0.58, 0.49 0.66, 0.86	0.48 1.02	0.38 0.56	0.53 0.55	0.42 1.02	0.48 0.43	0.75 1.47
Ratios to geometric mean of controls	1.5 3.0		0.90 1.36	0.73 0.74	1.02 0.74	0.80 1.36	0.90 0.58	1.42 1.95
Geometric mean of ratios			1.11	0.73	0.86	1.04	0.72	1.66
2	1.5 3.0	0.45, 0.14 0.38, 0.48	0.31 0.53	0.34 0.50	0.31 0.37	0.15 0.51	0.21 0.53	1.04 1.30
Ratios to geometric mean of controls	1.5 3.0		1.25 1.25	1.35 1.17	1.25 0.87	0.58 1.19	0.82 1.25	4.12 3.04
Geometric mean of ratios			1.25	1.26	1.04	0.83	1.01	3.54
3	2.0	0.39, 0.35, 0.47	0.53, 0.49, 0.21					0.63, 1.29, 0.79
Ratios to geometric mean of controls			0.95					2.15
Grand geometric mean of ratios ..			1.09	0.96	0.95	0.93	0.85	2.33
95 per cent limits			0.77–1.55	0.62–1.47	0.62–1.46	0.60–1.43	0.55–1.31	1.64–3.31

* The "adjuvants" were given as stated in Table I. The mice were killed 10 min. after receiving most "adjuvants," but 20 min. after receiving glycerol.

toxin, and morphine we were not aware of methods of estimation sufficiently sensitive for our purpose. Aird and Strait (1944), however, estimated cocaine in the nervous tissue of cats by a spectrochemical method ; they found that the amount passing into the brain was lowered by 31 per cent, and that into the cerebrospinal fluid by 40 per cent, in animals stained with trypan red. In our hands, unfortunately, the recoveries from mouse-brain were so low as to preclude us from using Aird and Strait's method to decide whether or not mortalities in mice accurately reflect the concentration of convulsant drug passing into the brain. Accordingly we turned to substances which can readily be estimated in the tissues.

Experiments with sulphanilamide and sulphanilic acid

These compounds were selected for test because, as we shall report in another paper, their behaviour *vis-à-vis* the blood-brain barrier is quite different. Sulphanilamide readily passes the barrier and in this respect may be compared with the basic dyes. Sulphanilic acid, on the other hand, normally penetrates the brain in only very small amounts, thus approximating to the acid dyes.

Table VI sets forth the concentrations attained in the brains of mice given these drugs combined with many of the " adjuvants " or with water. Owing to a misunderstanding of instructions, the mice receiving glycerol were given the undiluted chemical instead of a 50 per cent solution ; in consequence, the observations with this drug were repeated and appear in Table VII. Table VIII presents similar results for hexamine, Table IX for coal-gas and for ether, and Table X for trypan red and for neutral-red chloride.

In the experiments listed in Table VI, more than one level of dosage of sulphanilamide and sulphanilic acid was used, giving concentrations in the brain at two different levels. In the statistical analysis the quantities were first transformed to their logarithms, in order to assess whether or not the " adjuvant " drugs had different effects at the two doses, and to combine the effect at the two levels. No significant differences were found between the effects at the different levels of dosage. The mean of the logarithms is equivalent to the geometric mean of the original results, which fact accounts for the presentation of the entries in Tables VI and VII in terms

TABLE VII

EFFECT OF GLYCEROL ON CONCENTRATIONS OF SULPHANILAMIDE AND SULPHANILIC ACID IN THE BRAINS OF MICE

Amounts are given in mg. per 100 g. brain. Each represents an observation on a single animal.

Experiment	Sulphanilamide 2 mg. and		Sulphanilic acid 2 mg. and	
	Water	Glycerol (50 per cent (v/v))	Water	Glycerol (50 per cent (v/v))
1	6.0	5.6	0.84	1.14
2	5.4	6.8	0.29	0.75
3	5.7	7.7	0.30	0.83
	Ratio to geometric mean of control 1.17 95 per cent limits 0.91–1.50		Ratio to geometric mean of control 2.13 95 per cent limits 1.20–3.79	

of geometric means. For the sake of consistency the same transformation is used for the results of the experiments in Tables VIII–X.

From the mean ratios in Tables VI and VII, and the limits to be associated with them owing to experimental error, it is obvious that the only individually significant effects are for glycerol and 50 per cent (v/v) glycerol and sulphanilic acid. Glycerol has the apparent effect of doubling the concentration of this drug in the brain; owing to experimental error the true increase may be as low as 64 per cent or as high as 230 per cent. Since, however, it appears reasonable to combine the results for glycerol and 50 per cent glycerol, we obtain by so doing the following figures:

Sulphanilamide: mean increase 15 per cent; 95 per cent limits of error 1–31 per cent.

Sulphanilic acid: mean increase 128 per cent; 95 per cent limits of error 69–208 per cent.

We conclude therefore that glycerol has a significant adjuvant action with sulphanilamide also, though this action is less pronounced than that with sulphanilic acid.

For none of the other " adjuvant " drugs listed in Table VI do the ratios approach the level needed for significance. We can say, however, that their adjuvant action, if any, is not likely to exceed 25 per cent for sulphanilamide or 50 per cent for sulphanilic acid. The margin of error is greater for the latter owing to the higher relative variability between mice, probably associated with technical difficulties in estimating low concentrations of drug in the nervous tissues.

Table VIII shows that hexamine has no statistically significant effect on the concentration of sulphanilamide in the brain, whereas it causes a significant increase of about 22 per cent (95 per cent limits of error 5–43 per cent) in the concentration of sulphanilic acid.

From Table IX it will be apparent that coal-gas produces a significant reduction of 36 per cent (95 per cent limits 24–46 per cent) in the concentration of sulphanilamide in the brain, whereas ether has no significant effect. Both coal-gas and ether increase significantly the concentration of sulphanilic acid, by 150 per cent (95 per

TABLE VIII

EFFECT OF HEXAMINE ON THE CONCENTRATIONS OF SULPHANILAMIDE AND SULPHANILIC ACID IN THE BRAINS OF MICE

Amounts are given in mg. per 100 g. brain. Each represents the mean of the concentrations in a group of three mice. Hexamine was given as stated in Table I and the mice were killed 10 min. later.

Experiment	Sulphanilamide 2 mg. and			Sulphanilic acid 4 mg. and		
	Water	Hexamine 50 mg.	Ratio	Water	Hexamine 50 mg.	Ratio
1	4.84	4.14	0.86	0.92	0.94	1.03
2	4.43	4.67	1.06	0.39	0.65	1.67
3				0.57	0.64	1.12
4				0.65	0.78	1.19
Geometric mean of ratios			0.95			1.23

TABLE IX

EFFECT OF COAL-GAS AND ETHER ON THE CONCENTRATIONS OF SULPHANILAMIDE AND SULPHANILIC ACID IN THE BRAINS OF MICE

Amounts are given in mg. per 100 g. brain. Each represents the mean of the concentrations in a group of three mice. In the experiments with coal-gas mice were kept unconscious for a total of 10 min. immediately before and again immediately after injection of the sulphanilamide or sulphanilic acid; they were killed 30 and 20 min. after receiving these drugs. In the experiments with ether they were kept continuously anaesthetized from the time of receiving drug and were killed 30 or 20 min. later (sulphanilamide and sulphanilic acid respectively).

Experiment	Sulphanilamide 2 mg. and						Sulphanilic acid 4 mg. and					
	No treatment	Coal-gas	Ratio	No treatment	Ether	Ratio	No treatment	Coal-gas	Ratio	No treatment	Ether	Ratio
1	2.71	1.98	0.73	4.16	4.42	1.06	0.84	2.36	2.82	0.83	1.03	1.23
2	3.34	1.64	0.49	5.32	5.64	1.06	1.14	2.52	2.21	1.07	1.96	1.83
3	3.77	2.78	0.74							0.89	1.43	1.60
Geometric mean of ratios			0.64			1.06			2.50			1.53

TABLE X

EFFECT OF PRIOR ADMINISTRATION OF VITAL DYES ON THE CONCENTRATIONS OF SULPHANILAMIDE IN THE BRAINS OF MICE

Amounts are given in mg. per 100 g. brain. Each represents the mean of the concentrations in three mice. Dosing with dye as in Table II. The mice were killed 20 min. after receiving sulphanilamide.

Experiment	Sulphanilamide 2 mg. and					
	Water	Trypan red	Ratio	Water	Neutral-red chloride	Ratio
1	2.64	1.98	0.75	4.78	4.58	0.96
2	4.80	5.30	1.11	4.40	3.95	0.90
3	3.93	4.40	1.12	3.82	5.06	1.33
4	3.74	3.52	0.94	3.31	3.84	1.16
5	4.22	4.12	0.98	3.45	3.74	1.08
6	4.21	5.06	1.20	3.95	3.38	0.86
Mean			1.01			1.04

cent limits 93–224 per cent) and 53 per cent (95 per cent limits 24–89 per cent) respectively.

Prior treatment of mice with trypan red or neutral-red chloride (Table X) does not influence the concentration of sulphanilamide passing into the brain.

DISCUSSION

In these experiments we first examined the effect of administering a variety of drugs, etc., on the passage into the brain of a number of acid and basic dyes. For the reasons stated, we were not wholly satisfied with the results and we turned to

TABLE XI

SUMMARY OF THE EFFECTS OF " ADJUVANT " DRUGS AND VITAL DYES IN VARIOUS TESTS DESCRIBED IN THIS PAPER

" Adjuvant " drug or vital dye	Nature of observation				
	Passage of dyes into the brain	Mortality from convulsant drugs	Analgesic effect of morphine	Passage of sulphanilamide into the brain	Passage of sulphanilic acid into the brain
Adrenaline and pituitrin	Bismarck brown, neutral red, and methylene blue increased. Other dyes unaffected	General increase	Slight increase	No definite increase	No definite increase
Theocin ..	Bismarck brown increased. Other dyes unaffected	General increase	Decrease	No definite increase	No definite increase
Sodium lactate	No effect	General increase	No effect	No definite increase	No definite increase
Urethane ..	Neutral red and methylene blue decreased. Other dyes unaffected	Cocaine increased. Strychnine and picrotoxin decreased	Not tested	No definite increase	No definite increase
Histamine ..	Neutral red increased. Other dyes unaffected	General increase	Increase	No definite increase	No definite increase
Hexamine ..	No effect	General decrease	No effect	No definite increase	22 per cent increase
Glycerol ..	Chlorazol fast scarlet and brilliant vital red increased. Pontamine sky blue, disulphine blue, bismarck brown, neutral red, and methylene blue markedly increased	Strychnine and picrotoxin increased. Cocaine decreased	Slight decrease	15 per cent increase	128 per cent increase
Coal-gas ..	Bismarck brown, neutral red, methylene blue, and disulphine blue increased. Other dyes unaffected	Not tested	Not tested	36 per cent decrease	150 per cent increase
Ether-anaesthesia	Bismarck brown, neutral red, methylene blue, and new wool blue increased. Other dyes unaffected	Not tested	Not tested	No definite increase	53 per cent increase
Trypan red ..	Not tested	General decrease	No effect	No effect	Not tested
Brilliant vital red	Not tested	Strychnine and cocaine increased. Picrotoxin decreased	Decrease	Not tested	Not tested
Neutral-red chloride	Not tested	Picrotoxin decreased. Strychnine slightly decreased. Cocaine unaffected	Increase	No effect	Not tested

three convulsant drugs—strychnine, cocaine, and picrotoxin—and an analgesic—morphine—as indicators of passage through the barrier. Some of the results then obtained left us in doubt whether we had in fact modified the concentration of drug in the brain, or whether the effects observed demonstrated merely the lower (or higher) resistance of the animal to a combination of drugs rather than to a single drug. An attempt to resolve this quandary by spectrochemical estimation of one of the convulsants—cocaine—was unsuccessful, owing to low recoveries of the drug from the nervous tissue of mice. Finally, therefore, we used sulphanilamide and sulphanilic acid, which can be estimated in nervous tissue with a fair degree of accuracy. Table XI provides a concise summary of all the results.

In considering the action of the individual "adjuvant" drugs, etc., it seems appropriate to begin in each case with the results obtained with sulphanilamide and sulphanilic acid, as these rest on the relatively firm basis of chemical estimation.

Glycerol

The experimental findings leave no room for doubt that intramuscular injection of glycerol leads to increased concentration of sulphanilamide and of sulphanilic acid in the brain. The other results with this substance, except those pertaining to cocaine and morphine, accord with the belief that passage of drugs into the brain is favoured by the very violent redistribution of body-fluids which follows injection of glycerol (or of hypertonic saline). With sulphanilamide, and especially with sulphanilic acid, the increased concentration in the brain is greater than may be accounted for by mere dehydration of the nervous tissues; King (1942) has shown that glycerol causes the mean percentage of solids to rise to 24.8, as opposed to the normal 22.1.

Other "adjuvant" drugs

Apart from glycerol, no drug appeared to have anything like a uniform effect on the passage into the brain of the various test-substances.

Hexamine increased penetration by sulphanilic acid, but had no definite effect with sulphanilamide. Statistical consideration of the experimental data showed that, within the 95 per cent limits of error, any increased passage of sulphanilamide did not exceed 25 per cent, with of course the probability that it was much lower, while the actual figures obtained (Table VIII) did not suggest an appreciable increase. [A similar comment should be understood wherever no definite increase is recorded for sulphanilamide in what follows; with sulphanilic acid the probability was 1 in 20 or less of an increase of 50 per cent.] Hexamine diminished mortality from the various convulsants and left unaffected the response to morphine and the coloration of the brain with dyes.

The other "adjuvant" drugs produced no definite rise in neural concentration of either sulphanilamide or sulphanilic acid. Adrenaline and pituitrin, theocin and histamine each increased mortality from all the convulsants used but did not act uniformly with respect to morphine; they appeared to increase the passage of at least some dyes normally penetrating the blood-brain barrier. Sodium lactate increased mortality from the three convulsants while leaving morphine and dyes unaffected. Urethane decreased the effects of strychnine, picrotoxin, and two dyes but increased that of cocaine.

Coal-gas

Exposure to carbon monoxide had opposite effects with sulphanilic acid (sharply increased passage) and sulphanilamide (decreased passage). It clearly facilitated the entry into the brain of dyes which easily pass the barrier.

Ether-anaesthesia

During anaesthesia with ether more sulphanilic acid passed into the brain, but the concentration of sulphanilamide was unaffected. Dyes normally passing the barrier coloured the brain more deeply during anaesthesia.

Vital dyes

While decreasing mortality from all convulsants, trypan red did not influence the analgesic effect of morphine or retard the passage of sulphanilamide into the brain. Neutral-red chloride also failed to retard the passage of sulphanilamide; with convulsants and morphine it and brilliant vital red gave contradictory results.

General remarks

It is perhaps pertinent to inquire whether in the doses (near the maximum tolerated) given in these experiments, the "adjuvants" could have been expected to show an effect. If any effect were due solely or principally to a direct change in capillary permeability, which is almost certainly not the case with every "adjuvant" used, it should be possible also to demonstrate an effect in the skin. On the assumption that the volume of blood in the cerebral vessels is proportionate to that in the body generally (see *Tabulae Biologicae*, **1**, 126) it is possible to calculate that at any one time the greatest amount of "adjuvant" present in the cerebral vessels of the mouse is roughly one-fiftieth of the total injected. This amount of "adjuvant" contained in 0.05 c.c. saline was injected intradermally into the skin of a rabbit which 5 minutes later received intravenous trypan blue (Menkin, 1936). In the areas receiving theocin, urethane, and particularly hexamine increased localization of dye occurred; with histamine a zone of increased staining surrounded a pale central area; with sodium lactate the colour of the skin matched that of a control area injected with saline; while adrenaline and pituitrin constricted the vessels and led to greatly reduced coloration. This experiment demonstrated that, when applied locally, very small doses of many of the "adjuvants" may influence the permeability of capillaries of the skin, but we do not claim that the conditions in any way resemble those obtaining in our other experiments. Indeed, on general grounds it seems very unlikely that the effects described in this paper could all be due to simple changes in permeability of the capillaries of the brain. For instance, it is very unlikely that such drugs as adrenaline and pituitrin act by truly increasing permeability, and for this reason we have studiously avoided the use of the term "permeability" with reference to our experimental results. This point will be discussed more fully in a further publication dealing with the pathogenesis of equine encephalomyelitis and louping-ill; Broman (1941) has already commented on the misuse of terms by investigators of the blood-brain barrier.

A second question we have asked is whether we have established the optimal time-relation between the dose of "adjuvant" drug and that of the test-substance. Except glycerol, all "adjuvants" were given intravenously so that they would be

at their maximum concentration from the moment of administration, and be available immediately to influence the penetration of the test-substance already circulating in the blood stream. If, however, any effect of an " adjuvant " were an indirect one, it would not necessarily be achieved immediately. In order to investigate this point with one " adjuvant," we carried out a further series of experiments with theocin and sulphanilamide, in which we compared the effect of the former when given 15 minutes before, simultaneously with, and 10 minutes after the sulphonamide. The results did not differ materially in the three cases.

Finally, whether any of the results obtained with convulsants and morphine can be interpreted in terms of increased or decreased passage of these drugs into the nervous system, or whether as discussed above synergisms and antagonisms between drugs invalidate or render dubious the results obtained in this type of experiment, it remains a fact that a given " adjuvant " drug may exhibit quite different effects with even closely related test-substances. The clearest example in the present series of results is that of the influence of coal-gas on penetration into the brain of sulphanilamide and sulphanilic acid ; here, chemical estimation reveals opposite effects, in that the passage of the blood-brain barrier by sulphanilamide is decreased while that of sulphanilic acid is enhanced by one and the same procedure. Other similar if less striking differences occurring in the tests in which drug passing into the brain was estimated chemically cannot be discounted, since they also were based on direct measurement rather than on the interpretation of possibly complex phenomena.

Conclusions

Using dyes, convulsant drugs, morphine, sulphanilamide, or sulphanilic acid as test-substances, we sought to increase or decrease their passage into the brains of mice. For this purpose we administered adrenaline and pituitrin, theocin, sodium lactate, urethane, histamine, or hexamine intravenously, or 50 per cent glycerol intramuscularly. We also exposed animals to coal-gas or to ether, and stained others by repeated injections of vital dyes. None of the drugs or treatments had a consistent effect on all the test-substances. These findings recall the conclusion of Stern and her colleagues that measures increasing the penetration of one substance into the brain do not necessarily increase penetration of other substances more or less similar chemically or physico-chemically. Table XI summarizes concisely the effects of combining the different drugs and treatments with the various test-substances.

We have discussed the validity of the experiments in which convulsant drugs or morphine constituted the test-substances, and conclude that it is preferable to use in this rôle chemical substances the concentration of which in the brain can be measured.

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