THE FATE OF INJECTED 4:4′ DIAMINODIPHENYL-SULPHONE IN HUMANS AND GUINEA PIGS.

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During clinical trials of the value of 4:4′ diaminodiphenyl sulphone in the treatment of leprosy (Molesworth and Narayanaswami (1)), a considerable number of estimations have been made to determine blood sulphone levels. The method, which has been used, depends on the development of a purplish red colour by coupling the diazotised sulphone with N-(1-Naphthyl)-ethylene diamine and has proved satisfactory for blood. At the same time, it was considered to be desirable to estimate tissue and urine concentrations and an adaptation of the method employed for blood was tried for this purpose, and is described in detail below. It was, however, found that, although the colour, developed from extracts of venous blood which contained sulphone, was similar to that developed by standard aqueous solutions of the pure sulphone—namely a purplish red—the colour developed by extracts of tissues and by urine, after clarification with trichloracetic acid differed appreciably by being considerably more pink in shade. It was considered that this observation warranted further study which is now described. Some values of sulphone levels found in tissues of guinea pigs that had received a series of injections of sulphone in oil and in tissues obtained post-mortem from a few human cases, that had been undergoing sulphone treatment, are also recorded.

Estimation of 4:4′ diaminodiphenyl sulphone in tissue and in urine.

Reagents:

1N-HCl.

2N-HCl.

12% (w/v) solution of trichloracetic acid.

50% (w/v) sodium nitrate solution. This solution remains stable for several months if kept in a refrigerator.

1.5% (w/v) ammonium sulphamate solution.

0.1% (w/v) solution of N-(l-Naphthyl)-ethylene diamine hydrochloride. This solution should be kept in an amber glass bottle in a refrigerator.

Standard solution of sulphone.

Stock Solution: 0.05 g. of 4:4′ diaminodiphenyl sulphone was dissolved in 500 ml. distilled water. This solution remains stable for several months if kept in a refrigerator.

Working Solution: 20 ml. of the stock solution is diluted to 100 ml. with distilled water just before use. The diluted solution contains 20 μgms. of sulphone per millilitre.

(a) **Method for tissue.** A suitable, accurately weighed quantity of finely divided tissue (from 0.1 g. to 1.0 g. depending on the expected concentration of sulphone) was ground in a test tube with an equal quantity of clean, dry, acid-washed sand. The pulpy mass was macerated with 5.0 ml. distilled water and 5.0 ml. 2N-HCl and allowed to stand overnight in the regrigerator. The mixture was filtered through a dry filter paper and an aliquot (normally 5.0 ml.) of the filtrate was pipetted into another tube and 6.0 ml. 1N-HCl, followed by 4.0 ml. 12% (w/v) trichloracetic acid were added. The mixture was well mixed and filtered through a dry 9 cm. No. 5 Whatman filter paper. To 10 ml. of the clear, colourless filtrate, the following solutions were added, mixing well after each addition:—3 drops of 0.3% (w/v) sodium nitrate* after which the mixture was allowed to stand for three minutes; 3 drops of 1.5% (w/v) ammonium sulphamate and standing for a further two minutes; and finally 3 drops of 0.1% (w/v) N-(l-Naphthyl)-ethylene diamine hydrochloride. The solution was transferred to a colorimeter tube and was kept in the dark for twenty minutes to allow full development of the colour, which was then compared, in a photoelectric absorptiometer, with the colour developed by standard solutions, prepared from pure sulphone and submitted to similar treatment.

Simultaneously with the treatment of the samples, a " reagent blank " was prepared by stirring a similar quantity of

sand with 5.0 ml. of distilled water and 5.0 ml. 2N-HCl, and the mixture treated in the same manner as the extracts. Before taking readings with the photoelectric absorptiometer (we have found a Coleman Junior spectrophotometer very convenient), the scale* was set at zero against the " reagent blank."

It has also been our practice to prepare, at the same time, two standards containing known amounts of sulphone. These were submitted to the same procedure as that already described for tissue extracts after treatment with HCl and filtration. For the stronger standard, 1.0 ml. of working solution, (= 20 μgm. of sulphone) was taken; and for the weaker standard, 0.2 ml. (= 4 μgm. of sulphone) was used. In each case, the volume was made up accurately to 6.0 ml. with distilled water, 5.0 ml. 2N-HCl added, followed by 4.0 ml. 12% trichloracetic acid and the remainder of the method followed, as already described.

(b) **Method for Urine.** Owing to the higher concentration of sulphone normally found in urine, it was necessary first to prepare a suitable dilution, depending on the concentration of sulphone expected. Such dilutions varied from 1 part to 5 parts of urine diluted to 50 parts with distilled water. A suitable aliquot of the diluted urine, e.g. 1.0 ml. to 5.0 ml. was made up to 6.0 ml. with distilled water, 5.0 ml. 2N-HCl was added, followed by 4.0 ml. 12% trichloracetic acid and the same method was then followed as has been already described for tissue.

The colour developed by tissue and urine extracts.

When this method was applied to samples of tissue and of urine from cases that had been undergoing a course of sulphone injections,* it was found that the colour developed by the extracts, after diazotisation and coupling was in every case considerably more pink in shade than the colour developed from the standard solutions of sulphone. The absorption curves of coloured solutions of approximately equal intensity developed from tissue extracts, heart blood and from standard solutions of sulphone were therefore, compared. The absorption curves were determined on a Coleman Junior spectrophotometer as described below; the extracts were obtained as follows:—

(i) **Kidney Extract.** 1.0 g. of kidney tissue, in which the concentration of sulphone had been found to 7.5 mgm. of sulphone per 100 g. tissue, was ground with sand and submitted to the procedure already described. The

* **0.3% (w/v) sodium nitrite.** This must be made up fresh immediately before use, by diluting 0.3 ml. of 50% (w/v) solution to 50 ml. with distilled water.

* The logarithmic scale for optical density was used i.e.: " D " = —log.T (where T = percentage transmission).

* Full details of the method of treatment are given by Molesworth and Narayanaswami (loc. cit.). Normally, injections of 1.0 to 2.5 ml. of a 20% solution of sulphone in coconut oil have been given twice a week.

extract, prior to colour development, contained 45 μgm. sulphone in 15 ml.

(ii) **Heart Blood.** 1.2 ml. heart blood (oxalated), containing 3.3 mgm. sulphone per 100 ml., was diluted to 6.0 ml. with distilled water, treated with 2N-HCl and trichloracetic acid, as already described. The extract, prior to colour development, contained 40 μgm. sulphone in 15 ml.

(iii) **Standard Sulphone.** 2.0 ml. working standard (= 40 μgm.) were diluted to 6.0 ml. with distilled water and 5.0 ml. 2N-HCl and 4.0 ml. trichloracetic acid were added. This solution contained 40 μgm. sulphone in 15 ml. In each case, a " reagent blank " was made in a similar manner, omitting the tissue, blood or working standard.

To determine the absorption curves, readings were taken at various wavelengths of the incident light in the range 400 to 700 millimicrons. At each selected wavelength, the scale which shows the percentage of transmitted light was set at zero against the appropriate " reagent blank "; the reading for the coloured extract was then made. Readings were taken at intervals of 50 μμ in the region where transmission was high and at intervals of 5 μμ in the wavelength region where transmission was found to be at a minimum. The percentage light transmitted was plotted against wavelength and the absorption curves thus obtained are shown in Figures I and II. It appears from these curves that some change has occurred, involving in the case of the extracts from the kidney tissue and heart blood, a slight shift to the left, i.e. toward the violet, of the point of maximum absorption as compared with a solution of the pure sulphone. In the former cases, maximum absorption appears to occur at $\lambda = 545$ μμ, whereas maximum absorption of the colour developed by pure sulphone is found to be at $\lambda = 550$ μμ. An explanation of this observation cannot yet be given. It was, however, possible that partial acetylation or some other form of conjugation of the sulphone may have occurred in the body, such as is known to occur with sulphanilamide and some of its derivatives; an attempt was therefore, made to isolate the sulphone (or the conjugated derivative) after passage through the body.

Isolation of sulphone from Urine.

The sulphone concentration of tissues has been found to be comparatively small; this fact, together with the difficulty likely to be experienced in isolating the sulphone from tissue in a quantity sufficient for further study, led us to attempt to isolate the drug from urine. Smith, Jackson, Chang & Longenecker (2) have

described a method which they found to be successful for the extraction of sulphone from rabbit urine. This method has now been successfully applied to human urine. The method depends on the fact that the free sulphone base is comparatively insoluble in water, but is readily soluble in ethyl acetate, which has been found to be the most suitable organic solvent so far tried. From ethyl acetate solution, the sulphone can readily be extracted with dilute acid, from which the base can be precipitated by the addition of alkali and subsequently purified.

13,500 ml. of urine, collected from a case that had been undergoing sulphone therapy for several months, and which was estimated to contain 4.6 mgm. of sulphone per 100 ml., was made strongly alkaline with 40% (w/v) NaOH and, after cooling to about 0°C., was extracted—in batches of 1,500 ml.—with three successive 400 ml. portions of ethyl acetate. To the combined ethyl acetate extracts, anhydrous sodium sulphate was added in small portions, with shaking, until the rather persistent emulsion was broken, after which the extract was filtered, through a dry filter paper, using suction, and the sodium sulphate washed with a further small quantity of ethylacetate. The combined filtrate and washings were washed with a small quantity of distilled water and then successively extracted with 50 ml. portions of 1N-HCl. The combined acid extracts were filtered, cooled in a refrigerator, made alkaline with ice-cold 40% (w/v) NaOH and extracted with three portions of ethyl acetate. The total volume of the second combined ethyl acetate extracts was about 250 ml. After washing with water this was again extracted successively with five 15 ml. portions of 1N-HCl. Air was then bubbled through the combined acid extracts, under low pressure, for a few hours, to remove as much of the residual ethyl acetate as possible. The pale yellow solution cooled to about 5°C., was made just alkaline by the cautious addition of ice-cold, 40% (w/v) NaOH, after which the alkaline liquid was left to stand for forty-eight hours in the refrigerator. The yellow, semi-crystalline precipitate was filtered off, washed with a small quantity of ice-cold water and after carefully drying at 60°C., was found to weigh 775 mgm; this compares with the quantity of 621 mgm. of sulphone, estimated to have been contained in the quantity of urine used.

In estimating the sulphone concentration of this sample of urine by the method already described, the colour developed by the extract, after clarification with trichloracetic acid, had shown the pinkish-red shade already described. Accordingly before proceeding further with the purification of the crude material isolated

from the urine, a 10 mgm. portion was dissolved in 500 ml. of warm water, the solution filtered, and 2.0 ml. (= 40 μgm. crude material) and 3.0 ml. (= 60 μgm. crude material) of the clear filtrate were each made up to 6.0 ml. with distilled water, 5.0 ml. 2N-HCl and 4.0 ml. 12% trichloracetic acid added and the colour developed in the usual manner. The resulting colour was found to be purple-red and similar to that developed by solutions of pure sulphone. If, in fact, the pinkish-red colour that was developed by the original urine was due to the existence of a loosely conjugated derivative of sulphone, which had been formed in the body, it appeared that this had been decomposed during the process of extraction; and it was probable that the material that had been isolated from the urine consisted of unchanged 4:4′ diamino-diphenylsulphone.

In order to demonstrate this, the remainder of the crude material was dissolved in the minimum quantity of methanol, the solution shaken with a small quantity of decolourising charcoal, filtered, and evaporated to dryness in a desiccator under reduced pressure. The residue consisted of 120 mgm. of white, needle-like crystals, which had a melting point of 172°-173°C. Pure 4:4′ diaminodiphenylsulphone has a melting point of 175°-176° C. A mixture of the recovered material and pure sulphone melted at 173°-174°C. thus indicating that the material which had been recovered from the urine consisted of unchanged 4:4′ diamino-diphenylsulphone. In order to purify it still further, the crystals were dissolved in the minimum quantity of acetone and warm water was added until a trace of turbidity appeared. After standing in the refrigerator overnight, the solution deposited a mass of colourless, needle-like crystals, which, after drying, were found to have M.Pt. 173°-174°C. 8.6 mgm. of these crystals were dissolved in 100 ml. water and the sulphone content of the solution was estimated by the usual method, and found to be 8.55 mgm. of sulphone in 100 ml. solution. The material isolated from the urine was thus proved to be unchanged 4:4′ diaminodiphenyl-sulphone.

Discussion.

The above results have not explained the reason for the development of the pinkish red shade by extracts of tissue and by urine from cases that have received injections of sulphone. To exclude the possibility that extraneous substances in the tissue might be affecting the shade of the developed colour, samples of tissue and of blood, obtained post-mortem from cases that had received no sulphanilamide or sulphone prior to death were

examined, but with negative results. Furthermore, the addition of pure 4:4′ diaminodiphenylsulphone to such tissue and blood in vitro, prior to clarification with trichloracetic acid, produced extracts that developed the purple-red colour, similar to that developed by the pure sulphone.

It is tentatively suggested that a labile additive compound may be formed by 4:4′ diaminodiphenylsulphone in the body; and that this compound is decomposed, liberating the unchanged sulphone, when in the presence of dilute acid or alkali. It is hoped that it will be possible to investigate this point further.

PART II.

4:4′ diaminodiphenylsulphone levels as found in certain tissues of guinea-pigs and in human tissue.

Owing to the difference in shade between the colour developed by extracts of tissue and that developed by pure 4:4′ diaminodiphenylsulphone, it cannot be assumed with certainty that a comparison of the intensity of these colours provides an accurate measure of the relative concentration of 4:4′ diaminodiphenylsulphone in such tissues. However, it is probable that no great error is involved in assuming that the intensity of the colour is, in fact, proportional to the amount of pure sulphone in the tissue extract. On this assumption, a number of estimations have been made of the distribution of 4:4′ diaminodiphenylsulphone in the body tissues of guinea-pigs that had received injections of the sulphone in oil for some months. The results are shown in Table I. Results obtained from the examination of tissues obtained from four human cases that had been undergoing sulphone therapy for some months prior to death, are also recorded in Table II.

The results obtained in the case of guinea-pig tissues show that the highest sulphone levels are found in the kidney and liver. They are low in the brain tissue. A certain degree of localisation of the drug appears to occur in the skin and muscle tissues near the site of the injections.

In the case of human material, the highest levels are again found in the kidney and liver. Unfortunately, no values have been determined so far for human brain tissue. In one case, a surprisingly high level was found in nerve tissue. No explanation is offered. Further estimations will be made when suitable material becomes available.

Case No. 596/49 provided most anomalous results, since, although treatment with sulphone had been continuous for twelve months prior to death, only faint traces of sulphone were detected in the tissues, apart from the liver; and even here, the sulphone

level was extremely low. Examination of further material may possibly throw some light on this anomalous case.

TABLE I.

Distribution of 4:4′ diaminodiphenylsulphone, as found in the tissues of guinea-pigs.

Case Number: ...	275/49	445/49	563/49	591/49	595/49
Date:	28.4.49	13.7.49	29.8.49	5.9.49	12.9.49
Period during which sulphone injections had been given ...	Three months	Eleven weeks	Four months	Four months	Four months
Total amount of 20% solution of sulphone in oil injected during the period—in minims	118	63	99	105	111
Total weight of sulphone injected in grammes	1.392	0.743	1.168	1.239	1.310
Live weight of guinea pig in grammes ...	635	—	520	620	540
Total dosage rate, i.e. Total weight of sulphone in grammes per kilo live weight ...	2.19	—	2.25	2.00	2.43
	Concentration of Sulphone found, expressed as milligrammes of 4:4′ diaminodiphenylsulphone per 100 grammes of tissue.				
Tissue of Organ					
Heart	0.9	0.8	0.4	1.4	0.4
Lung	1.9	1.2	0.6	1.2	0.7
Spleen	2.0	2.0	1.1	1.9	1.2
Brain	0.7	0.5	0.2	0.3	Trace
Liver	3.4	2.7	1.5	2.2	1.9
Kidney	2.5	3.2	1.0	1.5	0.9
Skin (near site of injection)	2.0	5.5	4.1	180.3*	6.5
Skin (not near site of injection)	1.3	0.4	0.7	3.3	0.3
Muscle (near site of injection)	1.0	1.4	8.8	58.1*	36.1*
Muscle (not near site of injection) ...	0.7	0.8	0.5	1.1	2.0

* These results are extremely high. It is possible that this may be due to contamination of the tissue with the carbo-fuchsin dye used to mark the site of the injections; but every precaution was taken to avoid including any such stained tissue in the material used for analysis. It is also possible that diffusion of the drug has not fully occurred, resulting in a high local concentration.

	Concentration of Sulphone found, expressed as milligrammes of 4:4′ diamninodiphenylsulphone per 100 ml. body fluid.				
Urine	—	—	—	9.7	2.0
Heart blood	0.4	—	0.4	0.4	0.2
Peripheral blood ...	0.4	—	—	0.5	0.2

TABLE II.

Distribution of 4:4′ diaminodiphenylsulphone, as found in tissue obtained from human cases, that had been undergoing treatment with sulphone.

Case Number ...	157/49	498/49	542/49	596/49
Date	7.3.49	25.7.49	23.8.49	22.9.49
Period of treatment with sulphone prior to death	Three months	Three months	Five months	Twelve months
Total number of injections, each of 0.2 grammes, given as a 20% emulsion in oil	27	23	50	110
Total weight of sulphone in grammes injected	5.4	4.6	10	22
Cause of death ...	Pulmonary tuberculosis	General Toxæmia Leprosy	Cirrhosis of liver	Pulmonary tuberculosis

Tissue	Concentration of Sulphone found, expressed as milligrammes of 4:4′ diaminodiphenylsulphone per 100 grammes of tissue or per 100 ml. body fluid.			
Heart muscle ...	3.4	—	0.5	Trace*
Lungs	3.4	3.3	0.6	Trace*
Spleen	4.0†	2.8†	0.5†	Trace*
Liver	6.1	5.1	1.3	0.3*
Kidney	6.6	7.5	0.9	Trace*
Skin	4.9	3.2	1.6	Trace*
Sciatic and Ulnar nerves	2.5	3.2	36.6§	Trace*
Bone marrow ...	1.8	—	—	—
Heart blood ...	1.5	3.3	—	—

† In every case, the colour developed by extracts of spleen tissue tended to show a brownish shade, somewhat different from the pinkish purple shade developed by extracts of the other tissues.

§ This result is out of all proportion with other results obtained and no explanation can be offered. The possible presence was excluded of other

compounds, with a primary amine group, that would be likely to give rise to development of colour on diazotisation and coupling.

This case is remarkable, since from the history and period of time during which injections had been given, it was expected that the tissue levels would have been, at least, as high as those found in the earlier cases. However, only a trace of the drug was detected. Whether any inhibitory factor was present or whether, in fact, no appreciable amount of sulphone had been retained in the tissues could not be determined. It has, however, been decided to record these findings, in case a similar anomaly should be encountered elsewhere.

SUMMARY.

(i) Extracts of tissue and of urine from cases that have received injections of 4:4′ diaminodiphenylsulphone have been found to develop a colour, when diazotised, and coupled with N-(1-Naphthyl)-ethylene diamine, which differs slightly from the colour developed by solutions of pure 4:4′ diaminodiphenylsulphone.

(ii) Absorption curves of the coloured solution obtained from these extracts and solutions show that the former possess maximum absorption at λ = 545 millimicrons while the latter shows maximum absorption at λ = 550 millimicrons.

(iii) The possible existence in the body of a labile conjugated form of the sulphone is suggested.

(iv) A few 4:4′ diaminodiphenylsulphone tissue levels as found in guinea pig tissues and human tissues are recorded.

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FIGURE I.

ORPTION CURVE OF THE COLOURED SOLUTION OBTAINED
J EXTRACT OF KIDNEY TISSUE AFTER DIAZOTISATION AND
COUPLING WITH N-(1-NAPHTHYL)-ETHYLENE DIAMINE.

DNEY TISSUE CONTAINED
5 MGMS. SULPHONE
:R 100 GRAMMES.

EXTRACT CONTAINED
45 MICROGRAMMES
SULPHONE IN 15 ML.

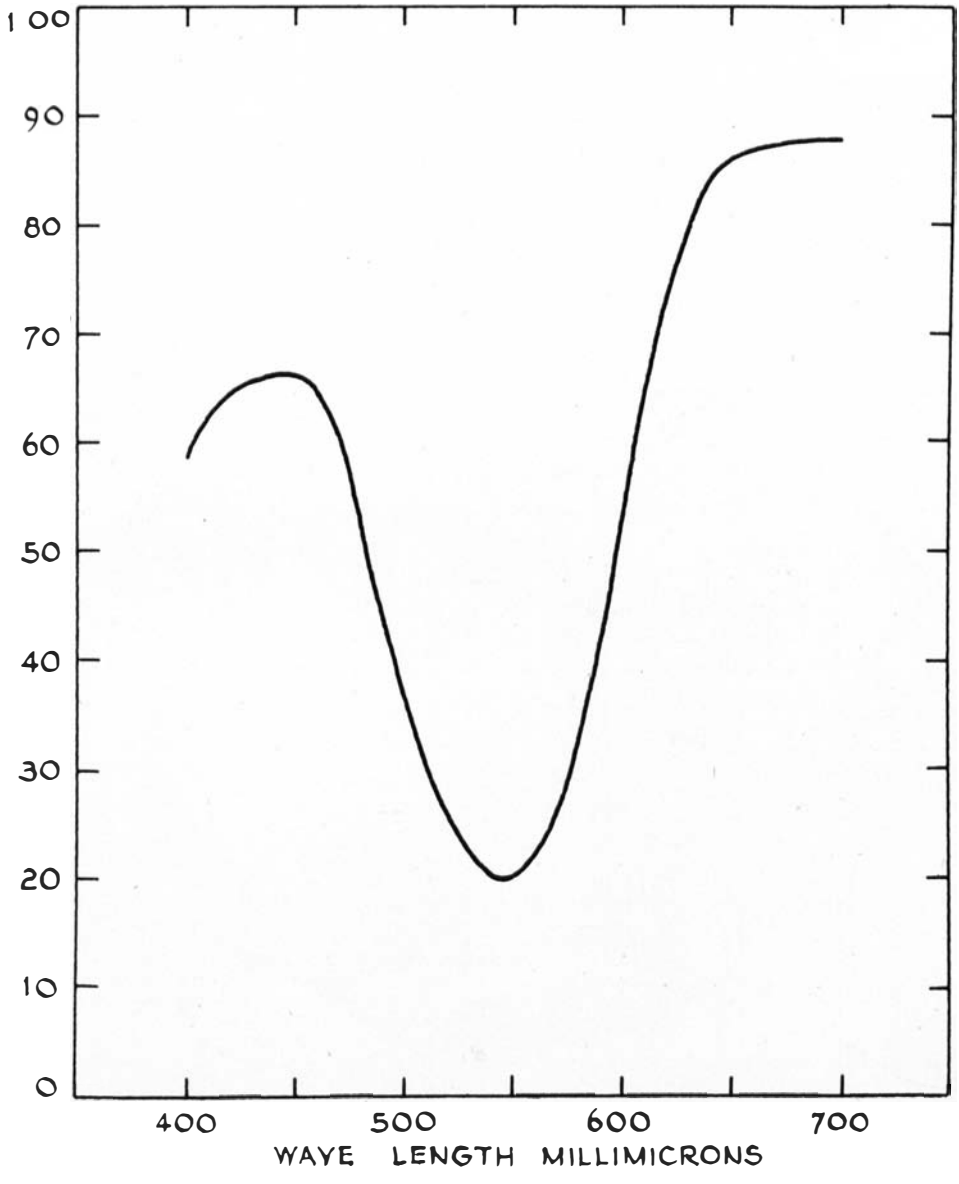

FIGURE II.

SORPTION CURVE OF THE COLOURED SOLUTION OBTAINED
)M A PURE SOLUTION OF 4:4' DIAMINO DIPHENYL SULPHONE
AFTER DIAZOTISATION AND COUPLING WITH
N-(1-NAPTHYL)-ETHYLENE DIAMINE.