sulfa drugs which proved to be effective in pneumonia. As soon as the efficiency of DDS against pneumococci in animals was discovered in these laboratories here, that substance was pressed into service in man. The blood level which was necessary to eliminate the pneumococcus in animals, i.e., somewhere about 5 to 7.5 mgm. per cent, is indeed toxic for man—terribly toxic. Doses of 1 to 2 gm. daily produced an acute hemolytic crisis on the third day, followed later by signs of central (cerebral) irritation. The pneumonia appeared to be successfully aborted.

The short explanation, then, of how the belief got established must be referred to the dose level. There is, of course, an extensive and well-documented literature upon the nature of the hemolytic poison which DDS is.

The new fact is that, provided one does not go above about 3 mgm. per cent in man, the acute hemolytic crisis seen with higher blood concentrations does not appear to intervene. Whether the second insidious toxicity which workers with laboratory animals have always seen with DDS, namely, slowly developing peripheral neuritis, will occur in man remains to be seen. It is quite clear that we must proceed cautiously with these studies in man, as indeed is being done.

I have been kept up to date, by correspondence and otherwise, with the work that is being done with this drug in leprosy. There is little doubt that this work, which seems to be started off by John Francis following observations of McEwen in cows, must be taken most seriously. I have been coming to the conclusion that we must find time to make a set-piece pharmacological study of DDS so that the clinical experimentation with it can be put on a sound basis.

[According to Dr. John Lowe, of the Nigeria Leprosy Service (personal communication), Buttle and associates of the Wellcome Research Laboratories, in their first report on experiments with DDS (Lancet 1 (1937) 1331), stated that after a single dose of 300 mgm. in man the blood had marked antibacterial properties. In a personal communication to Lowe, Buttle has said that a therapeutic trial was made in human beings suffering from acute infections, with doses of the order of 1 to 2 gm. a day. Because of the rapid production of methemoglobinemic and other toxic effects that treatment was promptly abandoned.—Editor.]

METABOLIC FATE AND DETERMINATION OF SULFONES

The contributions which follow have resulted from the inquiries addressed to various consultants, as related in a note in the editorial section of this issue. Some of the contributions were made in the form of memoranda, while others have been prepared from correspondence. That is particularly the case with the first item, which is a compilation of excerpts and rewrites of material from a correspondence to which he devoted extraordinary attention, he being actively in sympathy with the purpose of gathering light on problems which his own group had had to try to work out for themselves. As a matter of fact that helpful attitude was exhibited by all who were approached in this matter; and their contributions, severally and together, merit
careful attention. That there are many questions still to be answered, and some conflicts to be reconciled, will be evident. Any reader who, having considered the material in this symposium and the related editorial note, finds himself inclined to offer any comment on the matter or to submit any question not dealt with here is invited to do so. Insofar as practicable all such material will be submitted to our consultants and subsequently published in this section.—EDITOR.

From Dr. John Lowe, Specialist, Nigeria Leprosy Service, Uzakaholi, Nigeria:

The idea that the therapeutic effectiveness of the sulfone derivatives depends upon their being broken down in the body to the mother substance (DDS), held by most British workers, apparently arose with Francis and other veterinary workers in England who showed that substance to be especially effective in mastitis of cattle when injected locally. Although Buttle, who had given it by mouth (in 1-to 2-gram doses), had found it highly toxic in man they were convinced that it could be used safely by injection. The I. C. I. Pharmaceuticals people interested Cochrane when he was there in 1946, and he undertook an experiment by that route of administration; and they then interested Muir who suggested that I try it out in Nigeria, which I did—against all advice—by the oral route. The results obtained to date have been reported [J. Lowe & M. Smith, THE JOURNAL 17 (1949) 181; M. Smith, Leprosy Review 20 (1949) 78; J. Lowe, Lancet 1 (1950) 145]. Further reports are at present in press.

Contrary to the prevailing idea of what happens to promin and diason in the body was Brownlee’s conviction that sulphetrone is not degraded to DDS but acts as the whole molecule. However, he has recently intimated (personal communication) that some of what is absorbed from the gut may possibly be degraded, and that it may be the slow rate of degradation which is accountable for the lack of toxicity of the drug. Since it is antibacterial in the test-tube he also suggests that, if M. L. Smith and associates, of Bethesda, are correct in their belief that the amino group or groups must be free or potentially free to have antibacterial activity, it may be degraded within the bodies of the microorganisms themselves.

With the sulfone derivatives only a part of what is given by mouth is absorbed (about 50% of diason, we have found, and 20% or less of sulphetrone), and most of what is present in the blood or found in the urine is unchanged. Blood concentrations of them, estimated as whole molecules, range from 2 to 3 up to as much as 5 mgm. per 100 cc. Smith has determined free DDS in the urine of patients treated with these drugs, and he has also demonstrated it in the blood, but the amount of it present there is very small. Hence it is not surprising that after oral administration of DDS—90% or more of which is absorbed—the usual blood concentrations of around 1 mgm. per 100 cc. are accompanied by favorable therapeutic response.

The question of what happens when promin is given intravenously is in line with questions which have concerned us, although because of exchange difficulties we have not used that particular product. Johnson [J. A. M. A. 114 (1940) 520] reported that, whereas a dosage by mouth of around 2
gm. per day (70 kgm. man) would cause toxic symptoms, 20 gm. per day could be given parenterally without such effects. In the latter case almost all of the drug could be recovered from the urine unchanged, whereas in the former case only about 30% could be recovered and a part of that was in the conjugate form. Hinshaw and Feldman [J. A. M. A. 117 (1941) 1066] found that whereas oral dosage of 1.2 to 3.2 gm. a day caused anaemia, doses up to 16 gm. per day could be given parenterally—best intravenously—with little or no untoward effect. Evidently the toxicity on oral administration is due to breaking down of the molecule in the gastrointestinal tract, and quite as evidently only a small proportion of the drug given parenterally is broken down. It appears that very low concentrations of DDS are active.

It is interesting that the doses of the sulfones which can be given by mouth, before tolerance is established, run roughly parallel to their content of the sulfone radical (DDS, m.w. 248). Of diazone (m.w. 520), not more than 1 gm. can be given; of promin (m.w. 780), less than 2 gm.; and of sulpheterone (m.w. 892.5), about 2 to 3 gm.

It is also interesting to speculate on how much free DDS the sulfones taken by mouth could provide, if what is absorbed were all hydrolyzed. On the basis of 50% absorption, a 2 gm. dose of diazone could provide nearly 1000 mgm. of DDS; and allowing 20% absorption for sulpheterone, a 6 gm. dose could provide about 235 mgm. Since only a small part of what is absorbed and found in the blood and urine has actually undergone change, it follows that a dosage of 200 mgm. per day of DDS itself, with its nearly complete absorption, will supply more free active principle than when the derivatives are given in tolerated doses.

With the derivatives, all the evidence is that increasing the dose beyond a certain point does not result in a more rapid therapeutic response, and the same is probably true of DDS itself. The optimum daily dose of that substance is probably less and possibly much less than the 300 mgm. which we have been using. Daily administration of small doses seems more effective than correspondingly larger doses given twice weekly.

As for the Bratton-Marshall test, we first estimate the free sulfone radical, after which we can calculate the amount of the actual drug involved on the basis of its molecular weights. For example, a reading of 1 mgm. of sulpheterone per 100 cc. (DDS) would be equivalent to

\[
\text{per 100 cc. (DDS) would be equivalent to } 1 \times \frac{248}{892.5} = 3.14 \text{ mgm. of sulpheterone.}
\]

When we say that the sulpheterone level is 3 mgm. per cent, that would be true only if all the sulfone present were in the original form, and we know that is not so. Feldman [Harben Lectures, 1946] realized this when he spoke of "blood levels as far as they can be determined chemically." Actually, in the colorimetric determinations we employ scales which are calibrated from standard solutions, and their values could not be established theoretically. The readings with the sulpheterone disc of my Lovibond colorimeter have to be divided by 2 when we are testing for diazone, and by 5 for DDS, and those figures are not in the ratios of the molecular weights. Colorimetrically 1 mgm. per cent of DDS is the equivalent of 5 mgm. per cent of sulpheterone, and not the theoretical 3 mgm.

I quite agree that it is liable to be confusing when a table of blood-level findings shows, for example, 1.5 mgm. per cent in patients treated with tolerated dosage of DDS, and 4.0 or 4.5 mgm. per cent in patients similarly treated with diazone or sulpheterone. It sounds as if these last
should be the best. But it is to be realized that the 1.5 mgm. of DDS consists entirely of the active base, while the figures for the complex sulfones refer to their whole molecules, of which the DDS base is a minor part (4.0 mgm. of diasone = 1.9 mgm. of DDS, and 4.5 mgm. of sulphurone = 1.3 mgm.). It would not be acceptable to record blood levels on the basis of the mother substance only, for on the one hand much of the whole complex in the blood is apparently not degraded to DDS before being excreted, and on the other hand there is the possibility that the undegraded substances themselves are therapeutically active.

JOHN LOWE, M. B., F. R. C. P., M. R. C. P.

From Sister Hilarry Ross, Biochemist, U. S. Marine Hospital (National Leprosarium), Corrville, Louisiana:

In considering the behavior of the sulfone drugs in the body, the position of the amine group with respect to the aromatic (i.e., benzene) group is important. In organic chemistry we have ortho, meta, and para (or end) positions, as shown below. In formulas these positions may be indicated by name, or by the initial letter (o, m, p), or by number as in the fourth of the following diagrams.

For such drugs to be therapeutically active the amino (NH$_2$) group must be in the para position, as in sulfanilamide and diaminodiphenyl sulfone (DDS).

When any compound having the para-amino group is introduced in the animal body a portion of the drug is, theoretically, changed to a conjugated product by the substitution of an acetyl (COCH$_3$) group for one of the hydrogens in the amino (NH$_2$) group. This change in sulfanilamide is shown in the third of the formulas immediately above. Theoretically, acetylation takes place in the liver and, some say, possibly in other organs. Some compounds are acetylated more readily than others, and—also theoretically—acetylation may vary from day to day in the same individual under the same conditions.

Now, when we determine a sulfonamide or a sulfone in a solution or in the blood or urine we diazotize the compound (i.e., break it down and produce the—N:N—linkage) with nitrous acid; and then we couple the diazo compound so formed—which has to be in an acid solution—with N-(1-naphthyl)ethylenediamine dihydrochloride to produce a purplish-red dye which can be estimated by any colorimetric procedure (the Bratton and Marshall method). This reaction depends on the presence of an amino group substituted in the benzene ring, and will estimate any compound to which the sulfone is changed in the body and in which one amino group...
is intact. The acetylated portion of the sulfone or sulfonamide does not respond to this test because the amino group or groups are "blocked." Where there are two amino groups in a compound, sometimes only one of them is blocked and the other is free, as in NH₂SO₂NHCOCH₃. There is partial blockage here, showing that the whole radical does not necessarily have to be blocked.

Where there is conjugation (or blockage), this condition can be broken down by hydrolysis. This is done by adding hydrochloric acid and boiling for one hour. This releases the NH₂ that was bound, and then we proceed to determine the compound. Without the hydrolysis, it is "free" sulfone that is determined. The free sulfone fraction plus the fraction which has to be hydrolyzed to be detected is the total sulfone content. (Stated otherwise, the total concentration determined after acid hydrolysis, less that of free sulfone, is the amount which is conjugated.) Theoretically, the acetylated or conjugated portion is therapeutically inactive, and we never determine that fraction because the clinician is interested only in what is available for therapeutic effect. According to the manufacturers, promin, diasone and promizole are acetylated in the animal body, whereas promacetin and sulphetrone are not acetylated.

Now, there are other factors which complicate the matter. For one thing, some of the promacetin in the blood becomes bound to erythrocytic proteins in the laked whole blood and so is lost when they are precipitated; consequently plasma is used both in testing for that drug and in making up the standards of it. With whole blood 86 per cent is recovered, and with plasma 100 per cent. With promin about 90 per cent is recovered when plasma is used. Promizole, too, is bound to erythrocytic proteins to some extent.

It is the general belief that the diaminodiphenyl sulfone radical is the element of the sulfone derivatives which has therapeutic value. I believe that if all of the blood and urine analyses were based on the molecular weight of the compound administered against the molecular weight of DDS, then the blood and urine values for the different drugs would be very clear. What I mean is this: Sulphetrone has four benzene rings, with a lot of other elements. Promin has only two benzene rings, and two complex substituent groups. Two grams of sulphetrone would be equal, approximately, to one gram of promin in DDS content. However, before any such correlation can be employed practically, much more scientific work must be done. In my opinion a complete pharmaceutical study of the sulfone drugs has never been made.

At the present time different sulfones require modifications of the Bratton and Marshall procedure as regards the amounts of the reagents used, the acidity of some of the reagents, and the material used (i.e., plasma instead of oxalated blood in certain instances). We hope in future work to standardize the method so that only one need be used, with perhaps a factor for each drug applicable to a single curve. This will entail a great deal of work, because comparative tests of each drug will have to be made.

From Dr. M. I. Smith, National Institutes of Health, U.S.P.H.S., Bethesda, Maryland:

Dealing first with the question of what takes place in the test for
Correspondence

18, 2

sulfones, aromatic amines diazotize with nitrite in an acid medium, as follows:

\[
\text{NH}_2 \leftrightarrow \text{N=N} \leftrightarrow \text{N} = \text{Cl}
\]

The free bond on the nitrogen then combines with the coupling reagent to form a dye, as was described by Bratton and Marshall (J. Biol. Chem. 128 (1939) 537). The reactions are as follows:

\[
\text{SO}_2 \leftrightarrow \text{NH}_2 + \text{NaNO}_2 + \text{HCl} \rightarrow \text{SO}_2 \leftrightarrow \text{N} = \text{Cl}
\]

\[
\text{SO}_2 \leftrightarrow \text{N} = \text{Cl} + \text{H} \leftrightarrow \text{NH} - \text{CH}_2 - \text{CH}_2 - \text{NH}_2
\]

The free bond on the nitrogen then combines with the coupling reagent to form a dye, as was described by Bratton and Marshall (J. Biol. Chem. 128 (1939) 537). The reactions are as follows:

\[
\text{SO}_2 \leftrightarrow \text{N} = \text{Cl} + \text{H} \leftrightarrow \text{NH} - \text{CH}_2 - \text{CH}_2 - \text{NH}_2
\]

The dyes so formed by the sulfonamides are water soluble. Those formed by the sulfones are as a rule sparingly soluble in water, and erratic and low readings will result if they are not redissolved. This fact is apparently not sufficiently appreciated by workers in this field. To meet this condition we have used 20 to 30 per cent acetone to ensure complete solution of the sulfone dyes formed after diazotization and coupling. This step was described in an article (J. American Pharm. Assoc., Sc. Ed. 37 (1948) 461) in which—dealing with another but related type of compound—we pointed out that 10 to 15 minutes are required for the formation of the chromogen on diazotization and coupling, and that during this time much of the chromogen forms colloidal aggregates. For this reason 5 cc. of acetone is added in all cases just before reading, to redissolve the chromogen. It is not added earlier lest it interfere with the formation of the dye.

Sulfanilamide, \(\text{NH}_2 \leftrightarrow \text{SO}_2 \text{NH}_2\), is partly acetylated in the body to \(\text{NH}_2\text{CH}_2\text{CO} \leftrightarrow \text{SO}_2 \text{NH}_2\), which will not diazotize until the \(\text{CH}_2\text{CO}\) group is split off by acid hydrolysis at 100°C to restore the free amino (\(\text{NH}_2\)) group. Thus the blood and urine of animals receiving sulfanilamide give one value on direct diazotization at room temperature and another, higher, value after acid hydrolysis at 100°C. The blood and urine of rabbits and guinea-pigs receiving DDS give the same values on direct diazotization as after acid hydrolysis. The inference is that, at least in these animals, the amino groups in DDS are not acetylated in the body.

Promin, with substituents on both amino groups, should not diazotize at room temperature, since it has no free amino groups. Actually it does diazotize, though incompletely. Presumably the substituents are sufficiently labile to be split off to some extent in the weakly acid medium at room temperature. Upon acid hydrolysis they are split off completely. If promin administered by mouth were absorbed and excreted unchanged, analysis of the blood and urine should give one value on direct diazotization and a higher value after acid hydrolysis. Actually we only get one value after administration by mouth, indicating that we are no longer dealing with promin as such but with a substance having the characteristics of DDS. The same is true of diazone and sulphetone, which are regularly given
by mouth. This we believe is evidence of the degradation of these substances to DDS.

While these disubstituted sulfones are apparently metabolized to DDS in the body, it appears that the monosubstituted alkyl and hydroxyalkyl derivatives do not undergo such transformation to any appreciable extent, as we have stated in a recent publication [Proc. Soc. Exper. Biol. & Med. 71 (1949) 25; see abstract in The Journal 17 (1949) 356]. One such substance, 4-amino-4'-N-hydroxyethylaminodiphenyl sulfone (called, for short, hydroxyethyl sulfone, or HES), has been studied particularly [American Rev. Tuberc. 60 (1949) 62; reprinted in The Journal 17 (1949) 435-441], and the evidence indicates that it is not metabolized to DDS. Incidentally, after study of the pharmacologic and chemotherapeutic effects in laboratory animals this substance has been tried out in a small series of cases of pulmonary tuberculosis with no untoward effects and seemingly good results, and arrangements have been made for a trial of it in leprosy.

For complete evidence regarding the degradation of the sulfones to the parent substance in the body it is necessary to isolate and identify the metabolite as DDS. This we are doing now by use of a method described in the first of the articles referred to above. The method is based on the solubility of DDS in ethyl acetate, which is immiscible with water, and the solubility of the hydrochloride of DDS in water. It works well. Titus and Bernstein [Ann. New York Acad. Sci. 52 (1949) 719-728] have found evidence of degradation of promin and other disubstituted derivatives to DDS by a method similar in principle to ours in which a different solvent, methyl iso-butyl ketone, is used. With our method we have now proven that HES is not metabolized to DDS but to the glycine derivative, \( \text{NH} \rightarrow \text{SO} \rightarrow \text{NH-CH}_2\text{COOH} \). We are applying this method to several disubstituted derivatives of the promin type and some other monosubstituted derivatives of the HES type, and should have a clear-cut answer in due time unless we run into unforeseen difficulties.

From Dr. Leon A. Sweet, Director of Research, and Dr. Eugene H. Payne, of the Department of Clinical Investigation, Parke, Davis & Co., Detroit, Mich.\(^1\)

We are inclined to believe that when promin is given intravenously it is only partially broken down to diaminodiphenyl sulfone (DDS) somewhere in the tissues, whereas when given orally it is split much more completely in the gastrointestinal tract. If that is the case, one might of course wonder why the parent substance itself is not used for the treatment of leprosy or tuberculosis. It would not be readily soluble for parenteral use, but as suggested in the inquiry it could be so administered by suspending it in peanut oil. The early work with it indicated that it was too toxic for use by the oral route, but it is possible that that conclusion was due to the use of excessively large doses, as indicated by reports of its use in leprosy which are now appearing.

There is a further possibility which can be illustrated with promin,

\(^1\) A synthesis of two communications, one received direct as a result of an inquiry addressed to Dr. William H. Fuldman of the Mayo Clinic, referred by him to Dr. Sweet, and one received by Sr. Hilary Ross, of Carville, who had referred to Dr. Payne certain questions asked of her.
and that is the splitting off of the substituted group on one but not both of the amines, changing it from the first to the second of the following compounds.

\[(\text{CHOH})_2\text{CH} \cdot \text{OH} \rightarrow (\text{CHOH})_2\text{CH} \cdot \text{OH}^-\]

\[
\begin{array}{c}
\text{NH} \cdot \text{CH} \cdot \text{SO} \cdot \text{Na} \\
(1)
\end{array}
\]

We were convinced from our extensive work on sulfones that it is essential that one of the amine groups be free or potentially free in order for the sulfone to be active in tuberculosis or other bacterial infections. It is virtually impossible to prepare the second compound indicated above in pure form for study, and even if it were obtained it would tend, like promin, to break down in vivo to the parent diaminodiphenyl sulfone.

There are, however, monosubstituted derivatives of the character of (2), with one free amino group, which are stable. One of them is the hydroxyethyl sulfone (HES) to which attention has been drawn quite recently. We have found unbalanced compounds of this type to be effective in vitro and in vivo against tubercle bacilli. The more active examples tended to be poorly soluble in water, however, without showing compensating advantages.

The question of whether DDS is the active substance in the sulfones has been the subject of considerable speculation among research workers. Most of the sulfones that have been employed have been derivatives of DDS, which was the first sulfone studied; promin and the other derivatives were made in order to reduce its severe toxicity. It is quite true that DDS in vitro is highly active, much more so than any of its derivatives, and the supporters of the DDS-base theory hold that all derivative compounds owe their activity to their slow hydrolysis and release of DDS. It is also quite true that the compounds now widely used—promin, dianone and sulphetone—are readily hydrolyzed to that substance.

This explanation of the activity of these derivatives of DDS does not apply to such sulfones as promacetin (acetosulfone) and its associated compound, propiosulfone. Promacetin is the sodium salt of 4,4'-diaminodiphenylsulfone-2-sulfone-acetamide, and has the following structure:

\[
\begin{array}{c}
\text{NH} \cdot \text{CH} \cdot \text{SO} \cdot \text{N} \cdot \text{C} \cdot \text{CH} \\
(2)
\end{array}
\]

This compound and the propiosulfone referred to are not derivatives of DDS in the sense referred to above, although they have DDS as a central nucleus. Nor does promacetin break down in the body to give DDS, since
a sulfonamide group, such as occurs in the second ring, is not split off in the body. It is quite probable that promacetin acts as the entire molecule; or, at most, the acetyl group might be split off from the sulfonamide group. This compound meets the requirements for an active sulfone in that one benzene ring is unsubstituted other than having a free amino group para to the sulfone linkage, which is essential for activity. In our studies with a large number of sulfones we found that this rule always held. Extra substituents could be put into the second benzene ring, and the amino group of that ring could also be bound, but if both amino groups of diamino diphenyl sulfone were to be substituted the substituent would have to be loosely held as in the case of promin or diamidin (diasone).

Since promacetin is therapeutically effective without undergoing degradation, it is an evident fact that sulfones do not necessarily and always depend on DDS for their value, although it may be that some of the simpler derivatives gain their activity in that way.

From Dr. Elwood O. Titus, of the Squibb Institute for Medical Research, New Brunswick, New Jersey:

We have done no work on sulfone blood levels in humans and can say little about promin degradation in man. In the dog, however, there is apparently some breakdown of the promin molecule after intravenous injection. When it is so administered in a dosage of 244 mgm. per kgm. (equivalent to 17 gms. per 150 lb. (70 kgm.) man), about 79 per cent of it is recovered apparently unchanged in the urine after 7 or 8 hours. Little more is recovered up to 48 hours. In the same period about 2 or 3 per cent of the administered dose appears in the urine as diamino diphenyl sulfone (DDS). The rest has not been accounted for.

After the above dose of promin given intravenously the blood levels of diamino diphenyl sulfone reach approximately 2 micrograms per cc. within 3 to 5 hours, and then fall off slowly. Blood levels of unchanged promin measured in the same experiments fell gradually from about 200 micrograms per cc. at one hour to about 40 at five hours and 7 at twenty-four hours.

The method we have been using to differentiate between diamino diphenyl sulfone and its water-soluble derivatives will be published as part of a study of the metabolic degradation of that substance in dogs. Essentially, the method involves extraction into methyl iso-butyl ketone, recovery into acid from the ketone, and analysis by the usual Bratton and Marshall method.

A convenient procedure is the following: Dilute 5 cc. of whole blood with 5 cc. of water and allow about 5 minutes for laking. Shake this solution with 6 cc. of methyl iso-butyl ketone (Carbide & Carbon Chemicals Corp.) for 2 minutes and centrifuge. Transfer 4 cc. of the upper phase to a separate funnel and shake for 1 minute with 4 cc. of 1 N hydrochloric acid. Treat 3 cc. of the hydrochloric acid lower layer with 0.3 cc. of 0.1 per cent sodium nitrite (which should be prepared fresh daily). After 3 minutes, add 0.3 cc. to 0.5 per cent ammonium sulfamate, and after another 3 minutes 0.3 cc. of 0.1 per cent napththylethylene diamine dihydrochloride (Eastman Kodak Co.). After 10 minutes the color is read at 540 μμ. This procedure will determine diamino diphenyl sulfone with no interference from substances such as promin or sulphetrone.
If the water-soluble derivatives are to be determined, 8 cc. of the lower (aqueous) phase from the original extraction may be treated with 5 cc. of a mixture of trichloroacetic and hydrochloric acids. This is prepared by dissolving 12 grams of trichloroacetic acid in 100 cc. of 3N hydrochloric acid. After standing for several minutes the precipitated proteins are filtered off and a 3 cc. portion of the filtrate is treated with the Bratton and Marshall reagents exactly as outlined for diaminodiphenyl sulfone.

This procedure has proved satisfactory for both blood and urine. With the latter, the pH is usually adjusted to 7 before extraction. Urine will generally require considerable dilution.

In a study of absorption and excretion of diaminodiphenyl sulfone and several of its derivatives [Ann. New York Acad. Sci. 52 (1949) 719-728] it was found that in dogs DDS—which was more completely absorbed from the gastrointestinal tract than the other substances worked with—was excreted largely as a water-soluble degradation product or products. These products of DDS behave like promin in the above procedure, i.e., they are not extracted into the ketone; so there will always be some question about the analysis of the water-soluble fraction. Formation of such substances will probably not be a source of great error, however, except when the blood levels of diaminodiphenyl sulfone are rather high.

From Dr. A. C. Bratton, Jr., Director of Pharmacological Research, Parke, Davis & Co., Detroit, Michigan:

The following explains the chemical reactions occurring during the determination of primary aryl amines by the Bratton and Marshall method.

In general, this method is applicable to most of the chemical compounds containing a primary amine group (-NH₂) attached to an aromatic ring.

The first step in preparation of a sample of biological material for assay is to remove protein and other interfering substances by treatment with a protein precipitant, such as trichloroacetic acid, or extraction by means of an organic solvent of the material which is to be determined. The next step is to lower the pH to 1 or below. When sodium nitrite is then added, the primary aromatic amine reacts with the nitrous acid to yield what is called a "diazonium salt," a process referred to as "diazotization." The chemical reactions involved in this step are indicated below, illustrated by sulfanilamide:

\[
\begin{align*}
\text{SO.NH₂} & + \text{HNO}_2 \rightarrow \text{SO.NH₃⁺} + 2 \text{H₂O} \\
\text{NH₂.HCl} & + \text{SO₂⁺} \rightarrow \text{NH₂⁺} \cdot \text{Cl}^{-} \\
& + \text{SO₂⁺} \cdot \text{Cl}^{-}
\end{align*}
\]

It will be noted that I have indicated that sulfanilamide exists as a hydrochloride salt, which is the case when the solution contains hydrochloric acid; if the solution contains only trichloroacetic acid, it will of course be associated with the organic acid. More properly, sulfanilamide associates with the hydrogen ion to give a charged molecule, and the chloride or other anion also exists as an ion. The resulting diazonium salt I have indicated as ionizing into a positively charged ion (cation), and a negatively charged ion (anion). Some aromatic amines react with nitrous...
acid very slowly, and the reaction is liable to be incomplete. Many diazonium salts are quite unstable except at a very low temperature, and a prolonged delay in going through the remaining steps of the colorimetric assay may result in incomplete conversion to a colored dye.

The next step involves the destruction of excess nitrite by sulfamic acid. This is necessary because free nitrous acid would react with the coupling component used later in the assay. It is generally more convenient to use the ammonium salt of sulfamic acid, which in the acid solution behaves as though the acid had been used. As shown below nitrogen is formed, and it may occasionally be seen as bubbles of gas in the tube.

\[ \text{NH}_2\text{SO}_3\text{OH} + \text{NHO} \rightarrow \text{N}_2 \uparrow + \text{H}_2\text{O} + \text{H}_2\text{SO}_3 \]

The final step in the determination consists of reacting the diazonium salt with the coupling component, \( \text{N-(1-naphthyl) ethylenediamine dihydrochloride} \), to yield a colored azo dye. It is believed that the attachment of the azo group to the naphthalene ring is in the 4 position; although this has not been proved to my knowledge for this particular coupling component, it has been shown to be the case for closely related compounds and I think the assumption is a good one. The azo dye formed is pink in acid solution and yellow in alkaline solution. Since a yellow color is usually quite difficult to estimate accurately in the colorimeter, we of course prefer to use the pink color in acid solution.

Quite a few diazonium salts couple rather slowly, and incomplete color development can of course be due to incomplete diazotization, decomposition of the diazonium salt, or incomplete coupling of the diazonium salt with the coupling component. These possibilities should be explored for each new substance worked with, and if complete development of color cannot be obtained by modification of the various steps it is obligatory to set up a very rigid set of conditions with respect to reaction times and concentrations to be followed in all analytical work with that substance.

For diazotization to occur, the aromatic amine group must be unsubstituted under the test conditions; that is, the nitrogen must have two hydrogen atoms attached to it. Many aromatic amines are conjugated with acetate in the body to yield an acetyl derivative. This is the case with all sulfonamides in common use. For instance, sulfanilamide is excreted in the urine partly as the acetyl derivative, \( \text{N-acetylsulfanilamide} \). These acetyl derivatives of aromatic amines are usually stable in cold acid, and therefore do not react in the diazotization procedure. However, boiling a solution of such an acetyl derivative with acid or alkali results in splitting off of the acetyl group to yield the parent substance, which then of course will react.

It is common procedure to determine the amount of diazotizable amine in unheated filtrates of biological material, the quantity expressed as "free" drug. Another sample of the same material is hydrolyzed by boiling with acid and, after cooling, is subjected to diazotization and coupling.
This gives the "total" amount of the drug, which includes the "free" drug plus the amount of conjugated drug that is hydrolyzable under the conditions of the test. Because our information regarding the precise nature of this latter product is usually incomplete, it is customary and proper to refer to it simply as "conjugated drug." Although most primary aromatic amines are excreted in part as acetyl derivatives, it is entirely possible that small amounts of other conjugated forms might be formed in the body, hence the use of the somewhat vague term "conjugated material" until a complete biochemical search has been made for the various degradation products.

A complicating feature of promin and diazone—in which the aromatic amine groups of diaminodiphenyl sulfone are conjugated with rather complex groups to make soluble products—is that, although these groups remain attached to the amine groups at pH values close to 7, they are split off to a greater or lesser extent in acid solution at room temperature. Although most primary aromatic amines are excreted in part as acetyl derivatives, it is entirely possible that small amounts of other conjugated forms might be formed in the body, hence the use of the somewhat vague term "conjugated material" until a complete biochemical search has been made for the various degradation products.

Dr. A. J. Glazko of our laboratories has set up a tentative general procedure for colorimetric determination of sulfones in common use, promin, diazone (diamidin), promazole, promacin and diaminodiphenyl sulfone (DDS), based on the use of isopropyl alcohol for the purpose of insuring the retention in solution of the somewhat difficultly soluble dye formed in the reaction. That procedure follows as an appendix to this note. It will be observed that after addition of trichloroacetic acid for deproteinization, a 20-minute period of standing is allowed for clumping of the precipitated protein. During this period the solubilizing substituent groups of promin and diazone are hydrolyzed in part; equilibrium is essentially established in this time in the case of diazone, but promin reaches equilibrium more slowly and consequently a waiting period of one and one-half hours before diazotization is recommended. In the case of promacin, the acidity has to be increased by the addition of hydrochloric acid solely to allow for nearly complete reaction with nitrous acid.

Under these conditions, solutions of diaminodiphenyl sulfone (DDS) yield an amount of color equivalent to that expected if both aromatic amine groups should react. Although promizole has two primary aromatic amine groups, only one appears to react. In the case of promin (in solutions which have stood for one and one-half hours) all of one group reacts and apparently about 30 per cent of the second group. In the case of diamidin (which is equivalent in structure to diazone), all of one group reacts and apparently about 18 per cent of the second group. Of all these sulfones, only diaminodiphenyl sulfone and promizole appear to be determinable by this procedure with precise quantitative accuracy. The accuracy of determination of the others depends on rigid adherence to an arbitrary set of analytical conditions, and I am quite sure...
that even under best conditions there will be some variation because of their incomplete reaction.

The interpretation of colorimetric analyses on blood and urine of patients receiving sulfones is further complicated by the fact that what happens to these substances in the body has not yet been completely explored. In the case of diaminodiphenyl sulfone, for example, one might expect theoretically to find in the urine of treated patients a certain amount of the unchanged sulfone, probably a certain amount of the monoacetoxyl or diacetoxyl derivatives, and perhaps other degradation products. Titus, of the Squibb Institute, has made a preliminary report on some biochemical studies he has done with diaminodiphenyl sulfone in laboratory animals [see preceding note and an abstract, p. 293 this issue]. He found that in dogs considerable amounts of a highly water-soluble substance appeared in the urine, although he has not to my knowledge definitely identified the material. Presumably it is phenolic in character, for many aromatic substances are partially oxidized in the body to yield phenols, which are then conjugated with glucuronic acid or sulfuric acid to yield highly water-soluble materials. Whether this is the case with diaminodiphenyl sulfone remains to be seen.

Such materials would certainly be expected to have a different rate of excretion than the parent compound, and the degree of conversion in the body to such substances would of course influence the "blood level" of aromatic amines. More important, we have little knowledge of the chemotherapeutic activity of such degradation products, although in general I should suspect that they are of lower activity than the parent substance. As yet no practicable method has been devised for accurately and separately measuring the various derivatives of diaminodiphenyl sulfone that may appear in body fluids of patients receiving this drug. Any such method would certainly be so formidable as to prohibit its routine clinical use.

As has been said, if the colorimetric determination of promin—which has a dextrose sodium sulfonate group substituted on each of the aromatic nitrogen—could be carried out at pH 7 or above, theoretically little or no color would be produced. However, at the acid pH where the determination must be made there is considerable hydrolysis of this molecule to yield primary aromatic amine groups, which of course give rise to colored azo dyes. In the body, no less than seven or eight degradation products of promin might exist. Due to the instability to be expected from certain of these structures, the investigation of these possibilities would be a very formidable undertaking.

Many clinicians are inclined to speak very glibly of blood levels, without realizing the limitations of interpretation that should be kept in mind. Nevertheless, colorimetric determinations, imperfect though they may be, still offer useful information. One is able for a new drug to do a balance study on urine and feces and determine the degree of absorption after oral dosage. Furthermore, it is possible to learn over a period of time what apparent blood levels, determined by a specific procedure, are associated with a satisfactory therapeutic response and with toxic manifestations. In the case of the sulfone drugs, I feel it would be highly desirable in the literature to speak of "free aryl amine concentrations" in blood and urine,
Correspondence

perhaps expressed in terms of the parent substance, and likewise for "total ary amine concentrations." This phraseology, I believe, would avoid implanting in the minds of those not familiar with the limitations of interpretation, the idea that we know precisely what organic substances are present in those body fluids.

With regard to questions raised about the dihydroxyethyl derivative of diamino diphenyl sulfone, reported by M. I. Smith and associates, the structure shows that one of the primary aromatic amine groups is free, and this is the one which diazotizes and couples. They have pointed out that the hydroxyethyl group is not split off by the body; if it were, the second aromatic amine group of diamino diphenyl sulfone would be released for chemical reaction. Also, they found that acid hydrolysis does not split off the hydroxyethyl group. I may point out in closing that, when a methyl group is introduced on the nitrogen of certain aromatic primary amines, the body is able to demethylate the resulting compound, again releasing the parent molecule. There are no sulfones in common use at the present time which have a methyl substitution on the nitrogen. Probably the body is unable to remove the hydroxyethyl group.

I regret that there is not to my knowledge any single publication which would give a reasonably clear picture of the considerations involved in this general problem.

APPENDIX

DETERMINATION OF SULFONE DRUGS IN BIOLOGICAL FLUIDS

(Dr. A. J. Glazko)

The usual colorimetric method for the determination of the sulfone compounds involves diazotization of the primary amino groups, and coupling with the Bratton-Marshall reagent in the presence of an organic solvent to keep the colored complex in solution. Details of the procedure vary to some extent for the different sulfones, but these differences have been minimized so that one general procedure can be used for the determination of promethazine, promazine, promethin (propranol), promesin, and diaminodiphenyl sulfone (DDS). We have had no experience with sulphetrone.

The procedure described here is satisfactory for determining "free" unaltered sulfones, but it will not detect conjugated metabolites in which the amino groups are blocked unless a preliminary acid hydrolysis step is introduced. All colorimetric values are converted to sulfone concentrations by reference to color standards prepared under the same recovery conditions as the unknowns, thereby eliminating any need for separate recovery corrections.

Reagents.—1. Trichloroacetic acid, 15 gm. in 100 cc. water. 2. Hydrochloric acid, 6N solution. 3. Sodium nitrite, 0.1 gm. per 100 cc. water. 4. Ammonium sulfamate, 0.5 gm. per 100 cc. water. 5. N-(1-naphthyl) ethylenediamine dihydrochloride, 0.1 gm. per 100 cc. water. (This coupling reagent should be kept in the refrigerator in a dark bottle when not in use.) 6. Isopropyl alcohol, reagent grade.
Procedure.—1. Pipette 1 cc. of plasma or serum into a small flask containing exactly 15 cc. water.
2. Add slowly, with shaking, 4 cc. of 15 per cent trichloroacetic acid. Shake well.
3. Let stand 20 minutes. Then filter, using a hard paper such as Whatman No. 50. The filtrate should be perfectly clear.
4. Pipette 10 cc. of the filtrate into a clean test tube.
5. Add 1 cc. sodium nitrite reagent and mix thoroughly. Let stand at room temperature.
6. After 3 minutes add 1 cc. of the ammonium sulfamate reagent and mix thoroughly. Immediately thereafter, add 4 cc. of isopropyl alcohol.
7. Three minutes after the addition of sulfamate (step 6) add 1 cc. of the coupling reagent and mix.
8. After 10 minutes standing, the color intensities in the filtrates are compared with those in a set of standards prepared in the same manner.

Wherever possible a photoelectric colorimeter with green filters should be used for measurement. Promin, diamidin (diasone), promacetin and diaminodiphenyl sulfone should be read at 555-560 m
where accurate selection of wave length is possible, and promizole should be read at 540 m
In the absence of a suitable colorimeter, naked-eye comparisons may be made with a set of freshly prepared standards, using test tubes of uniform diameter.

For analysis of urine, one usually makes a preliminary dilution of 1:10, 1:50, 1:100 or 1:200, as required, followed by the preparation of a further 1:20 dilution in 3 per cent trichloroacetic acid as described in steps (1) and (2) of the procedure. The final concentration of drug should be about 0.5 mg. per cent in the filtrate.

Standard Solutions.—Stock solutions are made up to contain 50 mg. of sulfone per 100 cc. of solution. Diamidin and promacetin should be dissolved in water containing a small amount (100 mg) of sodium bicarbonate. Promin should be dissolved in water to which is added 0.5 cc. concentrated HCl per 100 cc., in which it will remain stable indefinitely. Promizole is difficultly soluble in water, and the weighed powder should be dissolved in 5 cc. of 55 per cent ethyl alcohol, and then diluted to 100 cc. with N/10 HCl.

The stock solutions are diluted with distilled water to obtain the "working standards." Usually, 10 cc. is transferred by pipette to a 500 cc. volumetric flask and diluted to mark. This solution, containing 10 micrograms of sulfone per cc., is used for the preparation of standards.

Recovery standards are set up as shown in the following table:

1 For promin determinations, allow the trichloroacetic acid filtrate to stand at room temperature for one and one-half hours to insure complete hydrolysis.
2 For promacetin determinations, add 2 cc. of 6N HCl to the 10 cc. of filtrate.
**Colorimetric Standards for Sulfones**

<table>
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<tr>
<th>Tube No.</th>
<th>Equivalent concentration of sulfone* (mg/100 cc.)</th>
<th>Volume of normal plasma or serum (cc.)</th>
<th>Volume of distilled water (cc.)</th>
<th>Volume of &quot;working standard&quot; trichloroacetic acid (cc.)</th>
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</tr>
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<td>1</td>
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<td>12.0</td>
</tr>
</tbody>
</table>

* In plasma or serum.

The trichloroacetic acid is added slowly with shaking, and the mixtures are allowed to stand for 20 minutes before filtration to insure complete deproteinization. Protein standards are allowed to stand in the acid for an hour and one-half before diazotizing in order to hydrolyze the compound. For color development 10 cc. of filtrate is taken, and in the special case of promecacin 3 cc. of 6N HCl is added to 10 cc. of the filtrate. The diazotization and coupling reactions are carried out exactly as described for the unknowns, and optical density measurements or color comparisons are made after 10 minutes standing at room temperature. If a photometric colorimeter is employed, it is convenient to read the standard drug tubes against the reagent-plasma or serum blank (tube No. 1 in the table). A standard curve is constructed, plotting instrument readings against drug concentrations. Per cent transmission readings are plotted on a logarithmic scale, while optical density readings are plotted on an arithmetic scale, yielding straight-line relationships with concentration. Such a standard curve should be rechecked from time to time, particularly when any changes in instrument adjustments or experimental conditions are made.

**Micromethod for sulfones**—The general procedures described here can be modified for the determination of sulfones in finger blood by adding 0.2 cc. of blood from a washout pipette to 7.8 cc. of water, rinsing the pipette with the diluted solution. This is allowed to stand for 10 minutes to permit laking, and then 2 cc. of 15 per cent trichloroacetic acid is added to precipitate the proteins. This mixture is filtered or centrifuged after 20 minutes standing, and 5 cc. is taken for analysis. The quantity of reagents used for color development is half that given for the macro procedure, but all other details remain unchanged. Standards are made up using 1:50 dilutions of normal whole blood, in place of the 1:20 dilutions of plasma or serum used in the macro procedure, and colorimetric readings of the unknowns are converted to sulfone concentrations from the standard curves.