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THE DETERMINATION OF TOTAL BODY WATER

OF UNANESTHETIZED ANIMALS

By

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CHAPTER I

THE PURPOSE OF THE PRESENT INVESTIGATION

Studies of water distribution under normal and pathological conditions have not until recently yielded results from which clear-cut conclusions could be drawn, mainly because there have been no practical methods for the quantitative determination of the water in the various fluid compartments of the body.

Early investigators were interested in locating the water depots in the mammalian body. Magnus (1900) found in his studies of saline-infused rabbits and dogs that the tissues (in general) were water reservoirs. In hydration studies on dogs, Engels (1904) observed in poorly controlled experiments that the muscle took up approximately two-thirds of the injected solution (0.6-0.9 per cent saline) and the skin, one-sixth; thus, he concluded that muscle and skin were the significant water depots. Tashiro (1926) noted that the amount of water withdrawn from the muscles of rabbits varied with each muscle; that the skin did not supply water until the muscle stores were depleted. Skelton (1927) studied the tissues of cats before and after injections of water, hypotonic and hypertonic saline. His results showed that muscle tissue received most of the fluid, but they did not indicate to which compartment (extra- or intracellular) it was chiefly distributed.

More recently the distribution in the extra- and intracellular compartments has been of chief concern. Gamble and his associates (1923, 1925, 1929, 1930, 1937) conclude from data on water and electrolyte content of blood and urine of the dehydrated man and rat that the interstitial fluid is the important phase which keeps the blood volume relatively constant; that the intracellular phase plays a secondary part in the exchange. Carl Schmidt (1850) in his classical study of the composition of the red blood cells concluded that in the diarrhea of cholera, water is drawn first from the extracellular and later, if necessary, from the intracellular phase.* Does this occur in all forms of dehydration, or does the dehydration accompanying diabetic acidosis, where there is a disturbance of acid-base balance, present another picture?

The fundamental mechanisms of many conditions in which there is a disturbance of water distribution are still a matter of conjecture. Eppinger (1917) believes that many of the edemas are associated with lowered thyroid efficiency. Elwyn (1929) has discussed edema in the light of the mechanism regulating water exchange, which he believes involves: (1) a nervous center in the diencephalon; (2) the reservoir organs; (3) the membranes in the tissues and the walls of the capillaries; and (4) the excretory organs. But, can one evaluate the relative importance of each factor without a knowledge of the exact distribution of water?

*It is interesting to note that Schmidt was one of the first to use these terms.

There is considerable evidence that the endocrines, particularly the pituitary, the adrenals and the pancreas have an effect on the control of water balance. Experimental data on thyroid and gonad control is less conclusive but nevertheless important. In which direction is water exchange affected by the thyroid hormone, from the intra- to the extracellular phase, or does it vary under different conditions? To what extent does redistribution of water occur in myxedema? What are the mechanisms involved? Which of the female sex hormones is responsible for water retention and in which compartment is water retained in the pre-menstrual period, in pregnancy, in menopause?

Other questions which confront the investigator concerning the mechanism of water distribution are the following: what changes occur during growth; in the monothermic animal, what part does water play in the regulation of body temperature under adverse conditions; and what occurs in shock.

Results of investigations of these problems would be much more specific if actual quantitative determinations of water in the fluid compartments of the tissues were possible. In one compartment, the blood plasma, this has been accomplished by a dye method, which was first advocated by Keith, Rowntree and Geraghty (1935) and later modified and improved by Hooper, Smith, Belt and Whipple (1920), Dawson, Evans and Whipple (1920), Gregersen, Gibson and Stead (1935), and Gregersen et al. (1937, 1938).

Another dilution method for the determination of fluid has been advanced by Crandall and Anderson (1934) and elaborated by Lavietes et al. (1936) and Gregersen and Stewart (1938). The evidence now indicates that the water available for the dilution of the anion, thiocyanate, approximates the extracellular fluid content of the body. Since the anion, however, is found in the red blood cell, this fact must be considered in the calculations of total extracellular fluid.

A method comparable to those previously mentioned has not been described for the determination of intracellular water. For this reason it seemed pertinent to investigate those substances which might be distributed throughout the total body water. Then by simultaneous determination of extracellular fluid and total body water, the distribution in the two phases could be calculated.

CHAPTER II

DETERMINATION OF TOTAL BODY WATER BY THE DESICCATION METHOD

It was Chaussier whom Claude Bernard (1859) described as one of the first (about 1830) to investigate the total water content of the body. Since Chaussier's original work is not available in this country, the account of Bernard must suffice:

> Ce rôle nécessaire d'éléments liquides dans tout organisme vivant donne un grand intérêt à une première question, celle de savoir quelle est la proportion d'eau que renferme l'organisme.
>
> Cette proportion a été recherchée chez l'homme par divers observateurs, et notamment par Chaussier, qui fit placer dans un four, a une chaleur assez peu considerable pour ne pas operer la carbonisation, un cadavre humain du poids de 60 kilogrammes. La dessiccation le reduisit à 6 kilogrammes. Les parties solides entraient donc dans sa constitution pour un dixième seulement, tandis que l'eau y était pour les neuf dixièmes. La momification, telle que la pratiquaient les Égyptiens, donne à peu près les momies ont été ramenées en général au dixième de leur poids. Ces chiffres, près chez l'homme, ne sauraient être généralisés et appliques à toutes les espèces animales. Chez les reptiles et beaucoup d'insectes, la proportion des liquides est beaucoup moindre; dans certain animaux, cette diminution de l'eau est telle que l'urine est solide et affecte une consistance pulvérulente. (pp.30-31)

Later investigators concluded that the high water and low solid content reported by Chaussier must have resulted from the fat loss which occurs long before "carbonisation". Bernard apparently accepted the figures, since they confirmed the reports on Egyptian mummies. He was concerned

primarily with the effect of hydration and dehydration of the organism on secretions and on the general physiological state.

Bischoff in 1863 performed a careful and detailed study of fresh cadavers, newborn and adult, from which he removed single organs and determined their water content by weighing and drying them at 100°C. For man he found 58.5 per cent of the body weight to be water and for the newborn, 66.4 per cent. The figures appear to be low. A possible explanation is found in the time of exposure of tissue during the process of dissection and weighing, which Bischoff does not discuss in his otherwise detailed report. It is to be noted also that he accepts the results of Dr. A. Friedleben for the water content of the skeleton on the basis of analyses of four bones of a 37 year old man. This is the only procedure which he did not carry out for himself. Many investigators rely upon this work, probably because of the detailed account of his method for obtaining the water content of single organs.

After determining the water content of all the bones of three skeletons and making approximately 100 other observations, Volkmann (1874) concluded that the water content varied in different bones. The average for a whole skeleton was 48.6 per cent (26.5 per cent higher than Bischoff's value), and agrees with figures of Hoppe-Seyler and Lukjanow, which Halliburton quotes in Schafer's textbook. This would indicate that the total water calculations of Bischoff were definitely low, since the skeleton makes up such a large portion of the

total body weight (16 per cent). But C. Voit (1881) in a discussion of "Bedeutung den einzelnen Nahrungstoffe" believes that Volkmann's figure is too high for the skeleton of adult man, since E. Voit found in the grown dog only 26.5 per cent water and in the young animal 63.4 per cent.

The conclusions of Volkmann are drawn from results of a large number of carefully controlled experiments. He was quite familiar with the work of previous investigators and very painstakingly avoided the errors in their procedures. He determined the water content of single organs, which he found difficult to dry unless they were chopped into small pieces. He used dry sheets of blotting paper to prevent the loss of exuding fat which he says came to the surface in the lean tissues and bone in the later stages of drying at a temperature of 100-120°C.

He says of Chaussier:

> Es war eine sehr ungluckliche Massnahme Chaussiers, zur Bestimmung des Wassergehaltes die ganze Leiche eines 60 Kilogr. schweren Mannes im Backofen zu trocknen, und verstehe ich nicht, wie der treffliche Claude Bernard, auf Grundlage dieses Versuches, die Wassermenge des menschlichen Körpers auf 90% veranschlagen konnte. Diese Angabe greift offenbar viel zu hoch, da selbst das Blut nur ungefahr 79% Wasser enthält. (p.218)

Volkmann's figure for total body water in man (65.7 per cent of body weight) agrees with those of Falck and Scheffer (1854) and of v. Bezold (1857) on animals. Falck and Scheffer,

in their investigation of the mechanism of thirst, very carefully studied water content of organs and tissues of "thirsty" and "not thirsty" dogs. v. Bezold was interested in water content of different animal classes and age groups. Seventy per cent of the total body weight he found to be the average figure for adult mammals. Amphibia and fishes were richer in water, not only because of their high water content in muscle, but also because the fat content of the body is low. Age and nutritional state he concluded to be important factors, inasmuch as the water content decreases while organic material increases with the development of each animal.

Volkmann remarks that his figure for man is the same as that of Bidder and Schmidt (1852) for cats. These men report data on "young" animals with no mention of age. Other work on the same species by C. Voit (1866) disagrees with the results of the above investigators, except when comparison is made with results on a cat starved for 13 days. Voit reports that the cats were very well fed for some time before the experiments. It is possible that the control animals were quite fat. As we know from the work of many investigators (v. Bezold, 1857, Fehling, 1877, Pfeiffer, 1887, Bouchard, 1897, Thomas, 1911, Moulton, 1923, and others) that fatty tissue is comparatively low in water (approximately 13 per cent in obese animals or man), it is not surprising to find that Voit's results differ from those of previous investigators. The differences are especially striking, when the figures are expressed as percentages of the total body weight (see Table I).

TABLE I

TOTAL WATER CONTENT OF ADULT MAMMALS
(Desiccation)

Date	Investigator	Mammal	Detail of Method	Result-per cent body weight	Remarks
1830?	Chaussier	man	intact-oven dried	90.0	loss of fat
1852	Bidder and Schmidt	cat	----	67.96	"jungen"
1854	Falck and Scheffer	dog	organs and tissues dried in air bath at 120°C. skeleton in sun heat for many days	65.675	
1857	v. Bezold	mammal	heated in air bath at 80°C. for 1 day, then dried at 120°C.	70.0	average result
1859	Moleschott	man	---	67.6	(quoted Quetelet's results)
1863	Bischoff	man	organs and tissues dried over water bath at 100°C.	58.5	used figure of Friedleben for bone
1866	Voit, C.	cat	dried single organs at 100°C.	58.1	fed very well for 2-3 months (fat?)
		cat		64.9	starved for 13 days
1874	Volkmann	man	organs and tissues dried at 100-120°C.	65.7	

TABLE I (continued)

1877	Fehling	rabbit	---	65.1	
1884	Krause	rabbit	organs and tissues	68.8	(see Skelton, 1927)
1887	Pfeiffer	dog	organs and tissues dried in oven at 100°C.	58.2	fat
		dog		62.3	
		rabbit		60.3	fattened for one month
		rabbit		63.7	starved for 13 days (Note: brain and spinal cord were not included.)
1897	Bouchard	man	series of formulae based on cylindrical form of man	66.0	
1904	Engels	dog	organs and tissues in oven	65.98	average for 6 animals no food and water for 4 days
1908	Rubner	rabbit	---	66.99	
		mouse		69.78	
1911	Inaba	rat	skin, tissues dried in air bath at 100°C.	70.91	
		mouse		70.37	
1911	Thomas	cat	removed skin and intestinal tract-cooked body until bones light and loose-then dried on water and air bath separately at 100°C for 1-2 days	66.65	did not include skin or G. I. tract
		dog		68.45	

TABLE I (continued)

1915	Donaldson	rat	intact-oven dried at 96°C.	67-68.5	
1917	Hatai	rat	body with g.i. tract removed dried at 95-98°C. for 1 week	65.3	
1923	Moulton	cow	---	71.5	age-2 years estimated on fat free basis

Since 1874 the water content per se has not been a subject of controversy. Investigators and those writing textbooks or discussions on water balance have accepted the data which was obtained from animals sacrificed for the purpose. Later work was concerned with the factors which effected differenees in water content of tissues and whole bodies. Fehling (1877) was interested in the "Physiology of Placental substance Exchange". He believed the sensible way to approach the subject was to study the composition (water, dry substance, ash, fat and protein) of fetuses. In his investigation of human and rabbit fetuses he found a marked increase in dry substance and a marked decrease in the total water content during the stages of development. He cites Bischoff's figures for adult man and his own for the rabbit.

Krause in his "Anatomie des Kaninchens" discusses the physiology of the systems and includes data on the water content of many tissues. The figures had been obtained partly from his own experimental work and partly from that of others.

Pfeiffer (1887) found that fat content of dogs, rabbits and hens was an important factor in the distribution of water in the tissues. He also observed that fatty tissue in thin animals was higher in water content than the same fatty tissue in fat animals. Unfortunately, he excluded the brain and spinal cord in his total analyses. Nevertheless, his results explain the variations in Voit's data on different nutritional states. Much later work by Bozenraad (1911) shows that the water content of fat tissue in the obese man

is considerably lower than in a thin subject, and from this he attempts to explain the wide variations in the literature. Schirmer in 1921 observed in his studies of the subcutaneous, mesenteral and kidney capsule fat of rabbits, man and ducks that the water-rich fat tissue of the thin and emaciated subject contains much more connective tissue than that of the water-poor fatty tissue of the obese.

On the assumption that man is cylindrical in form, Bouchard in 1897 derived formulae for the composition of anthropometric segments. His results indicate that for persons of average weight the composition of the body can be calculated from the fixed protein present in a segment, but that for obese subjects his formulae did not hold. His paper is quite vague and incomplete and shows a lack of consideration for the reader, since he has no references and indicates no method of analysis. However, it has been mentioned here to show that in the quite different approach of Bouchard, obesity is an important factor in the estimation of total body water.

Engels (1904) investigated the importance of tissues as water depots in the body. His dogs received no food or water for four days prior to experimentation. His results on the water content of these partially dehydrated "control" animals and the wide range in their body weights would lead one to believe that some were obese dogs. (This was not the case for his "experimental" animals, whose weights did not differ so widely.) If this were true, it is not surprising that his figures agree so closely with those of Volkmann on man.

Independently, Rubner, Inaba, Thomas, Donaldson and Hatai studied the growth problem and its effect upon the composition of bodies of many animal groups. Rubner investigated the changes in the quantities and ratios of substances during growth. Inaba observed the variations in warm and coldblooded animals of different age groups. In certain mammals, Thomas (cat, dog), Donaldson (rat) and Hatai (rat) noted the rapid changes in water, ash and fat content during the period when young animals double their birth weight. A comparison of the results obtained by these investigators should be accepted with reservation, since Rubner does not describe his method, Thomas does not include the analysis of skin and alimentary tract, and Donaldson and Hatai dried the whole bodies of rats.

In order to determine the effects of age and abnormal development on the composition of animals, Moulton has discussed and tabulated his own results and those of others by transposing all figures on water content from a total solid to a fat-free relationship. In cattle and man, on which he made his original observations, he found a decrease in water content up to five months after birth. At this time a comparatively constant level of water, proteins and salts is reached which he calls "chemical maturity" of cells. It becomes apparent that his high values are the result of exclusion of fat tissue from his analyses and calculations.

A study of the experimental procedures of the investigators cited shows that tissues were dried at temperatures ranging from 95° to 120°C. (see Table I). Benedict and

Manning (1905) very carefully investigated the effect of heating substances at 100°C. to determine water content indirectly as the loss of weight of material. According to these authors, 110°C. is too high, since physical and chemical changes, such as volatilization of solids (especially nitrogenous material), absorption of oxygen and abstraction of moisture from the air during process of cooling and weighing occur at this temperature. Some volatilization will also occur when material is dried at 100°C. They conclude from their observations that drying in high vacua without heat is the best method. Shackell in 1909 advocated an improved method of desiccation. The sample is mixed with clean ignited sand to prevent shrinkage and exposed to high vacuum over concentrated sulfuric acid in a desiccator.

CHAPTER III

ESTIMATION OF TOTAL BODY WATER FROM OSMOTIC PRESSURE CHANGES

Since 1920, the approach to the study of the quantity and the location of water in the animal body has changed. Interest is now centered around the distribution of fluid between the extra- and intracellular phases, and the factors influencing and regulating the interchange of fluid between these compartments and between blood and tissue spaces.*

Much work has been reported on the distribution of sodium and potassium in the body in relation to fluid exchange. According to Katz (1896), Gamble et al. (1923), Van Slyke et al. (1923), Peters and Van Slyke (1931), Peters (1935) and others, cell membranes or some restraining characteristic of the media prevent the free transport of these electrolytes between extra- and intracellular compartments.

From a variety of experiments (pyloric and intestinal obstruction, fasting, etc.) Gamble and his collaborators, McIver, Drake, McKhann, Ross and Tisdall, concluded that

*Many of these studies have been done on anesthetized animals without regard for the possible effects of the anesthetics. Heller and Smirk (1932) have studied the effect of some anesthetics, among them ether and chloroform, upon water exchange in rabbits and rats. They found a definite delay in absorption and excretion of water, but they could not account for any shift in water in the tissues examined, namely, liver and muscle. In dogs McAllister (1937) has demonstrated that plasma volume is reduced approximately 12 per cent during ether anesthesia.

whenever there is an increase or decrease in potassium or sodium there is usually a sufficient gain or loss in intracellular fluid (when the ion is potassium) or extracellular (when sodium) to maintain the osmotic pressure of cellular fluid in equilibrium with that of plasma and to keep the electrolyte concentration constant in extracellular fluid.

By measuring the osmotic pressure changes of the blood, following intravenous injection, Hetherington (1931) found in cats anesthetized with amytal that approximately all the water in the body is "available to take part in osmotic interchanges between blood, tissues and cells". The "available" water is 59 per cent of the body weight. She believes that this figure, which is lower than other values for cats (see Table I), is indicative, not of the presence of "bound" water, but of the significant part of the total weight represented by the skin and hair. The possibility that sodium chloride might be stored in the skin without an increase in water deposit was overlooked, a fact which would certainly prevent an otherwise more marked osmotic pressure change (Nencki et al., 1894, Wahlgren, 1909, Padtberg, 1910).

About the time Hetherington published her report there was much controversial evidence about the state of water in the organism. In 1930, A. V. Hill reviewed and discussed the problem and presented results of vapor pressure studies on blood and muscle, which indicated that 97-98 per cent of the water is "free". He also re-interpreted the results of Overton, who was the first to point out the probable existence

of "bound" water. Moran (1930) confirmed the work of Hill by a dilatometric freezing-out method for mammalian muscle, but Jones and Gortner (1932), using the same method, reported the presence of 0.7-4.7 gm. "bound" water in each gram of gelatin. Later in the same year Greenberg and Greenberg (1932) tabulated the results of 10 investigators for one colloidal system, gelatin. They discussed the virtues of each method used and concluded that the question should not be determined by the colligative properties of a system which shows such wide deviations from an ideal solution. They believed that ultrafiltration, which permits the passage of crystalloids and the solvent but not the colloidal constituents, yields a filtrate in which the concentration of the crystalloids becomes a measure of the solvent water. Thus they found that biological colloids have very little, if any, water associated in a "bound" form.

More recent reports in the literature indicate that there are still those who conclude that not all water in cells is solvent. Parpart and Shull (1935), who compared the results of different investigators, noted that the values for the quantity of solvent water in mammalian red blood cells were not consistent. Since ethylene glycol does not dissolve in or adsorb onto the solutes within the cell, they studied the distribution of this non-electrolyte between cells and an environment of salt solution. The results indicate that only 50 per cent of the cell volume is available for glycol dilution. This they assumed to be solvent water.

Macleod and Ponder (1936), who conclude from their own work with glycol that all cell water is solvent, criticized the work of Parpart and Shull on the basis of faulty technique in analysis and the possibility that some oxidizable material might be present in the fluid surrounding the cells. They also stated that the erythrocyte can not be considered a perfect osmometer as Parpart and Shull maintain, since it undergoes much less change in volume than would an osmometer under the same conditions. Hetherington's work, therefore, supports the views of Hill, Moran, Greenberg and Greenberg, and others on this question.

After the ingestion of sodium chloride, Felix Arden (1934) has found that the blood chloride level was highest at the end of two hours, and showed that fluid sufficient to make up 52 per cent of the body weight took part in the osmotic equilibration. This figure is unreliable, for calculations were based on an assumed blood volume figure and an approximate excretion figure.

Recently Gilman (1937), in an investigation on the effects of hydration and the distribution of fluids upon thirst, has estimated the osmotic pressure changes of the blood of unanesthetized dogs following the administration of urea and sodium chloride. This change was of such magnitude for urea that the fluid taking part in the osmotic equilibration made up 65.0 per cent of the body weight, and for sodium chloride 68.0 per cent. These figures confirm those by the desiccation method on the same animal (see Table I).

Results of these and other experiments based on osmotic pressure changes have been tabulated in order to show that the figures vary over a wider range than those obtained by desiccation (see Table II).

Recent controversy over the distribution of potassium and sodium ions and their diffusibility through membranes can be partly attributed to the variations in the chemical technics used, partly to the difference in animal species and in the types of cell membranes studied. The ability of one or both of these cations to pass through the red blood cell has been demonstrated by Yannet, Darrow and Cary (1935). In the adjustment of osmotic equilibrium following intraperitoneal injections of glucose and sodium chloride, these investigators using the method of Schechter et al. (1933) found in the erythrocyte of the dog the passage of sodium as well as a shift in water. In the monkey and rabbit, there was a shift of water only. They conclude that in the cells which show a preponderance of sodium (dog), the membranes are permeable to this ion; but for cells in which potassium is the predominant base (monkey, rabbit) neither the sodium nor the potassium ion will pass through the membrane. Thus they attribute this difference to the particular electrolyte configuration of red blood cells of the animals. Kerr (1926, 1929) finds that changes in potassium concentration of the red blood cells of the dog, sheep and cow are of such magnitude that they cannot be accounted for by shifts in water, but only by actual diffusion of the potassium.

TABLE II

TOTAL WATER CONTENT OF ADULT MAMMALS

(Osmotic Pressure Changes)

Date	Investigator	Mammal	Detail of Method	Result-per cent body weight	Remarks
1931	Hetherington	cat	osmotic pressure studies injection of NaCl	59.0	possible retention in skin
1934	Arden	man	ingestion of NaCl determinations of blood and urine chloride concentrations	52.0	calculations were based on assumed blood volume and an approximate excretion figure
1935	Lavietes, D'Esopo and Harrison	man	formulae on electrolyte	70.0	
1936	Harrison, Darrow and Yannet	rabbit	dried at 105°C.	75.0	rabbits dried intact
		dog	chloride distribution	64.0	tissues dried
		monkey	for extracellular fluid	65.0	tissues dried
		monkey	estimation	72.0	monkey dried intact
		same animals	based on distribution of sodium and potassium ions	56-72	
1937	Gilman	dog	osmotic pressure studied		
			injection of urea	65.0	
			injection of NaCl	68.0	

Fenn and his co-workers (1934, 1936, 1937) have observed that frog, rat and cat muscle cells are permeable to potassium and sodium; the cells exchange potassium for an equivalent amount of sodium when the muscles show voluntary activity, or when they have been stimulated electrically. In his review of "Electrolytes in Muscle " (1936), Fenn discusses many different aspects of water and electrolyte exchange. One criticism he makes of the past work in the field is that muscles per se have not been analyzed in every experiment. His most recent contribution (on cat muscle) (1938) shows: that the loss of potassium is balanced by a gain in chloride; that the gain in water is greater than that accounted for by the gain of isotonic saline; that reversal occurs after recovery in a few hours.

Using intraperitoneal injection of glucose, Darrow and Yannet (1935) have observed internal fluid shifts between extra- and intracellular compartments of dogs, monkeys and rabbits under conditions when there were presumably no changes in total body water. The shift in water attended the changes in concentration of the extracellular electrolytes. When extracellular electrolyte was removed, there was a migration of water into the cells, as evidenced by results of analyses of electrolytes and protein in blood cells and serum. When the electrolyte was increased in the interstitial fluid there was movement of water toward this phase. Thus it was concluded that changes in total extracellular electrolyte content were responsible for water movement.

In a study of salt and water exchange between muscle and blood, Hastings and Eichelberger (1935, 1937) found that the extracellular phase makes up 17 per cent of the dog's normal fat-free skeletal muscle and the intracellular phase, 83 per cent. Their figures were calculated from a series of formulae derived on the original assumptions that all the chloride and most of the sodium are in the extracellular phase and that normally there is no transfer of potassium, sodium or chloride across the membranes separating the extra- and intracellular compartments. It has been pointed out that in the dog 13 per cent of total muscle sodium is found in the intracellular phase (Harrison, Darrow and Yannet, 1936). Fenn (1936a) demonstrated that muscle fibers of the rat and cat, when exercised by electrical stimulation, are permeable to the three ions, sodium, potassium and chloride. Quite recently he has observed the same phenomenon during voluntary contractions of the rat muscle. Hastings and Eichelberger did find, however, by equilibration in vitro that death or injury to the muscle will effect a change in permeability. This fact might be presented in defense of their hypothesis, except for the fact that Fenn et al. have recently (1938) found a reversible exchange of these ions during recovery from stimulation of intact cat muscle.

In different animals the distribution of each electrolyte between cells and plasma is quite variable (Abderhalden, 1897, 1898, and Kerr, 1937). Studies have been carried out for the most part on blood and muscle and not on other structures,

which have quite different functions from the two tissues mentioned. Glandular organs which secrete these electrolytes should show very interesting data concerning their intra- and extracellular electrolyte concentration.

CHAPTER IV

A SEARCH FOR SUBSTANCES WITH WHICH TO DETERMINE TOTAL BODY WATER BY THE DILUTION METHOD

Three salient points should be considered in this analysis: the substance must diffuse freely through cell membranes; it must be non-toxic in the doses used and preferably pharmacologically inert; it must be one which can be determined accurately in body fluids. Likewise, the metabolism of the compound, its fate in the body and the rate of excretion of end-products are significant.

In the literature one can find numerous experiments which have been performed on isolated cells and tissues in the investigation of cell permeability. Information regarding the diffusible substances used in such studies (immersion of cells in solutions and perfusion of solutions through glands) has aided considerably in the search for those substances with the required specifications; but while the list is extensive it is also discouraging. Most investigators present information concerning a single confined aspect of the problem on one type of tissue. However, by a systematic approach, the substances in question can be retained or discarded as potential agents for determining total body water by the dilution method. They will be discussed from this point of view in the following pages. A summary of pertinent data is presented in Table III.

TABLE III

SUBSTANCES DISTRIBUTED IN BODY WATER

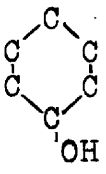
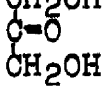
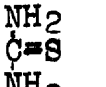
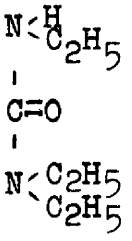
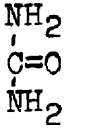
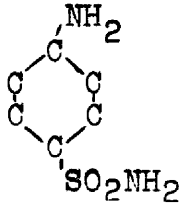
Substance	Mol.Wt.	Chemical Nature	Structural Formula	Permeability	Pharmacology	Remarks
Ammonium salts	(of inorganic and organic acids)			Single cells (Stewart and Jacobs)	acidosis	disturbs acid-base balance
Alcohol ethyl	46	lipoid sol. (sl.)	C_2H_5OH	Single cells (Stewart)	metabolized	
Deuterium oxide	20	not lipoid sol.	H_2H_2O (D_2O)	Mammalian erythrocytes (Parpart)	----	too long for equilibrium D ions replace some H ions
Phenol	94	lipoid sol.		Mammalian cells (Marenzi, Barac)	toxic	
Glycol propylene	76	neutral in reaction	CH_3CHOH CH_2OH	Single cells (Jacobs and Stewart)	toxic in dosage about 30%	distributed in organs and tissues
Glycol ethylene	62	neutral in reaction	CH_2OH CH_2OH	Single cells (Jacobs)	toxic	
Dimethylurea	88	lipoid sol.	$NHCH_3$ $C=O$ $NHCH_3$	Submaxillary gland cells (Amberson and Hober)	hypnotic	

TABLE III (continued)

Dioxyacetone	90	not lipoid sol.		Submaxillary gland cells (Amberson and Höber)	metabolized	
Thiourea	76	lipoid sol. (sl.)		Fish cell (Clarke and Smith	hypnotic	very slowly penetrates mammalian cells(Jacobs)
Triethylurea	146	lipoid sol.		Plant cells (Overton)	toxic	
Urea	60	not lipoid sol. (sol. in water and lipoids- Gad-Andresen) neutral in reaction		Mammalian cells (Table V)	inert, except in high conc.	distributed equally in
Sulfanilamide	172	neutral in reaction sol. in water and lipoid		Mammalian tissues (Marshall)	inert, except in large doses or after prolonged administration	distributed in organs and tissues

Diffusible Substances

Ammonium salts. In permeability studies on Arbacia punctulata eggs, Stewart (1931b) found that ammonium salts of fatty acids, the molecules of which upon hydrolysis yield ammonia and free undissociated fatty acid, will penetrate the cells easily. After penetrating the cell membrane these molecules unite again to form the salt. Since apparently all cells are permeable to ammonia and the lower fatty acids, Jacobs (1922) believes that such salts may penetrate any type of cell. Grossfeld (1936) in his permeability studies of tissue cells from heart, vessel, stomach and liver of the chick embryo has confirmed Jacob's conclusion.

When ammonia and its salts are administered to the intact animal the resulting effect is complex. Some investigators report a conversion of the inorganic salts into urea (Keith and Whelan, 1926, Bourdillon, 1937); others report the development of acidosis (Haldane, 1921, Baird, Douglas, Haldane, and Priestley, 1923, Adolph, 1925); still others the production of diuresis, which is attributed by Haldane to acidosis, but which according to Keith and Whelan occurs only when there is a shift of water. Adolph finds that the ingestion of inorganic ammonium salts produces acidosis in man and dog with the elimination of more nitrogen than can be accounted for in the salt administered, while the organic salts cause retention of nitrogen.

Gamble et al. (1924) have shown in man that these acid producing salts cause increased excretion of fixed base in the urine; that changes in plasma are accompanied by removal of body water which is closely proportional to the increased excretion of fixed base. With such disturbances in the acid-base balance, the maintenance of a physiological state of water balance is questionable. Therefore, although the isolated cell shows remarkable permeability to ammonium salts, the administration of these substances produces effects upon the intact animal which do not warrant their use in the present problem.

Alcohol. In permeability studies on Arbacia eggs, Stewart (1931a) found that alcohol penetrates the cells at the same rate as water and more rapidly than urea. That alcohol appears in all tissues of mammals following its administration has been shown by a number of investigators (Chittenden et al., 1898, Grehant, 1899, 1907, Nicloux, 1899, 1900a, 1900b, 1913, Hanzlik and Collins, 1913, Carpenter and Babcock, 1917, Carpenter, 1929, Haggard and Greenberg, 1934a, Le Breton, 1936, Harger et al., 1937, and others).*

A high percentage (85-90 per cent) of administered alcohol is oxidized in the body (Atwater and Benedict, 1899, Mellanby, 1919, Haggard and Greenberg, 1934b, Le Breton, 1936,

*Ford, 1872, Landsberg, 1904, Maignon, 1905, claim that alcohol is normally present in the body in very small amounts. Although this is generally accepted, according to Harger and Goss (1935) it is questionable, since there are no methods specific for tissue ethanol.

and others), but there is little agreement as to the factors controlling the rate of oxidation.

From a careful study of the reports of Mellanby, Widmark, Haggard and Greenberg, Mehrtens and Newman, Le Breton, and Harger et al., it can be observed that the alcohol distribution ratio ($\frac{\text{blood conc.}}{\text{total body conc.}}$) is dependent upon the method of administration, upon absorption and oxidative processes. By extrapolation of the time concentration curve (blood) back to the time when the alcohol was given by mouth, Widmark (1933) believed that he could determine the ratio, but he neglected to consider that oxidation occurs during the period of absorption, so that his blood levels are too low at the start. With intravenous injections Haggard and Greenberg (1934b) obtained a much lower ratio, even though they did not take into account the oxidation which occurs between time of injection and the withdrawal of arterial blood (8 to 15 minutes). The difference in the two ratios indicates the rapidity with which oxidation can occur during and following the absorption of alcohol. It is interesting to note that the results of the latter workers indicate that water equal to 50 per cent of the body weight of dogs is available for the dilution of alcohol within 15 minutes after the injection.

Mehrtens and Newman (1933) found the concentration of alcohol in the red blood cell to be 10-15 per cent lower than that in the plasma when alcohol was injected intravenously in man. One might expect to find this difference because the water content of the cell is lower than that of

the plasma. The records of these investigators show that the blood level drops sharply during the first and second hour after injection. They attribute this to rapid diffusion in the tissues. After this time the spinal fluid level parallels that of the blood, but the concentration of the spinal fluid is higher by 10 to 20 per cent. At least one-half of the difference can be explained by the difference in water content of the two fluids, and the rest by the rate of distribution and oxidation of the alcohol in the spinal fluid.

Harger, Hulpieu and Lamb (1937) in a study of the rate of distribution of alcohol following oral administration found the size of the dose and the presence or absence of food in the gastro-intestinal tract to be responsible for the variations in the rate of absorption and distribution. The results obtained with oral and intravenous administrations showed that there is a lag of one to two hours in diffusion to muscles; after this time the substance was distributed in approximately the same proportion as the water content of the tissues which they examined (brain, blood, liver, spinal fluid, muscle, stomach and intestine).

Even though much evidence seems to indicate that alcohol is distributed according to the water content of the tissues, its oxidation prevents the further consideration of the substance for the quantitative determination of total body water.

Deuterium oxide. Recent permeability studies of the mammalian erythrocyte show that these cells are permeable to deuterium oxide. However, the heavy water molecule penetrates the beef, rat and sheep cell at a much slower rate than does the water molecule (Parpart, 1935, and Brooks, 1935). According to Krogh and Ussing (1936) heavy water diffuses through all tissues, and from their analyses they conclude that after 4 to 8 days, the concentration is the same in all the free water of the body. However, during this period there is an exchange between the H and D atoms in the water and the organic materials of the body. The heavy hydrogen atom serves as an index for following the synthesis as well as the analysis of organic material in the mammalian body (Schoenheimer and Rittenberg, 1935, Rittenberg and Schoenheimer, 1937a, 1937b, Krogh and Ussing, 1936 and Smith et al., 1936). The Danish workers have found certain factors which they believe are significant in the exchange of H for D atoms in the body: (1) the formation of new fat; (2) proteins taking part in muscle contraction with reaction of one or more groups in the molecule which permit exchange of the isotopes. This protein activity, they believe, is responsible for the slow rate at which muscle reaches equilibrium with other tissues and fluids. Hansen and Rusting (1935) are of the same opinion. In 1934 certain investigators, McDougall et al. and Hevesy and Hofer, reported that deuterium oxide was distributed equally throughout the body water; but Hansen and Rusting found that although the distribution was equal

in two mother rats and their fetuses and in six rabbits given different doses, the dilution of the heavy water was such that results were 10 per cent lower than they should have been on the basis of total water in the body. Their explanation that a quantitative exchange of hydrogen with deuterium in the tissues is responsible for this low figure seems to be in keeping with the more recent observations.

Barbour and Trace (1936) in a pharmacological study of heavy water, found that mice after drinking 25 per cent deuterium oxide for several days showed 11.5 to 18 per cent saturation. This indicates distribution in 60 to 70 per cent of the body weight, but this figure includes the deuterium oxide in chemical combination in the tissues as well as that distributed in the body water.

It seems highly probable that this substance is equally distributed after the H and D exchanges are completed, but to what extent these changes occur and how long it takes for equilibrium in each animal is not predictable. Such a substance can not, therefore, be included in the present investigation.

Phenol. Prompt and uniform distribution of phenol in the fluids and tissues of dogs is observed following its intravenous administration. The conjugated forms of this substance are normally found in blood in small quantities (Marenzi and Banfi, 1936, Schmidt et al., 1937, Barac, 1937b). Tauber (1878) gave dogs phenol by mouth and found that the fraction "oxidized" increased with a decrease in the size

of the dose. De Jonge (1879) recovered only a trace of monophenol in urine of dogs, but he did recover its conjugation products which showed slight differences from those in man.

Pelkan and Whipple (1922a, 1922b) in reviewing and investigating the absorption, conjugation and excretion of phenol and p-cresol, have contributed much to an understanding of the physiology of phenols. They conclude from their work that injected phenol is probably distributed uniformily in the tissues, but that it soon disappears, some of it being oxidized in the body fluids and liver, and the rest being conjugated in the liver with sulfuric and glucuronic acids. Their results show these conjugated phenols to be maximal in concentration in one to two hours. The conjugated forms show uniform distribution in tissues and are slowly excreted in six to twelve hours. When phenol is ingested, the results are somewhat different. This substance according to Sollmann, Hanzlik and Philcher (1910) produces a local slowing of intestinal circulation and prevents steady absorption. Some reservation must be made in drawing conclusions from these experiments, because the methods of analysis are not specific for monophenol or its conjugated forms.

Barac (1935a, 1935b) and Barac and Lambrechts (1936), being fully aware that the chemical analyses do not warrant definite conclusions, found in searching for a specific method that phenol determined by the ultraviolet spectrographic analysis of blood was equally distributed between plasma and cells.

This was observed when trichloracetic acid was used to clear the solution, but was not the case when the tungstic acid procedure was followed. Barac's later (1936) analyses of blood, tissues and urine of dogs following the intravenous injection of phenol in doses of 25 mgm. per Kg. showed that phenol is uniformily distributed within a few minutes and completely removed from the blood in two hours. He observed that the quantity excreted in the urine represented only four to five per cent of the total injected. Because he found prompt distribution in an eviscerate preparation, he concluded that the disappearance could not be attributed to fixation in the kidneys, liver or gastro-intestinal tract. It is significant to remark that he makes no mention of conjugated products in this report. Marenzi (1936) criticizes this work of Barac and states that conjugation can occur without the function of the kidneys or liver. In the following year (1937a, 1937c) Barac reported that conjugation did occur and that it must take place in all the tissues since the blood of eviscerate and nephrectomized dogs contains the free and conjugated phenols.

If complete recovery of injected phenol in the form of free and conjugated products can be obtained from the urine, it might be possible to include this substance in the prospective materials for further investigation. However, the margin of safety for dosage of this compound is small. Barac gave very small doses subcutaneously, 25-100 mgm. per Kg., as larger doses produced a marked fall in the blood pressure.

Propylene glycol. Other substances which show wide distribution when injected into the tissues are the glycols. These and many other polyhydric alcohols have been compared in permeability studies by Jacobs (1931a, 1935) Jacobs and Stewart (1932) and Stewart and Jacobs (1932, 1933, 1935). Cell membranes of Arbacia eggs are quite permeable to ethylene and propylene glycol. In the studies of the two glycols these investigators found that the propylene penetrated the cell membranes more rapidly than the ethylene compound. This fact, they believe, can be explained in a way similar to Overton's observations in 1895, namely, that alkyl substitution for the hydrogen atom in a molecule increases its permeability. Recently a quantitative method for the analysis of propylene glycol in body fluids and tissues has been described by Newman and Lehman (1936, 1937). They found, in a comparison of blood concentration curves after gastric and intravenous administration in dogs, that absorption and diffusion occurred rapidly in the body. The disappearance of this dihydric alcohol was proportional to its concentration in the blood. From experiments on nephrectomized dogs they concluded that combustion of the glycol, which they believed accounted for a part of the disappearance in blood, took place at a constant rate. In a study of the excretion of the substance they found for dogs and rabbits that the recovery in urine (12-45 per cent) was greater, the higher the blood concentration. Since diuresis followed large doses, excretion is probably dependent upon the rate of urine flow.

No mention is made of a search for conjugated products in blood or urine. They accept the assumption of Seidenfeld and Hanzlik (1932) that glycol in moderate doses is burned to carbon dioxide and water. The latter examined urine only microscopically. Miura (1911) found qualitative evidence of a glucuronic acid conjugation product in the urine of rabbits after injections of propylene glycol. If this does occur, then low recovery of the unconjugated glycol in the urine does not necessarily indicate that a high percentage has been oxidized. In a report of the toxicity of the two glycols, ethylene and propylene, Hunt (1932) found no poisonous action from the latter. Propylene glycol was well tolerated by animals whose livers and kidneys had been impaired by other poisons. He suggests that this substance may have a true food value, but makes no mention of metabolic end products.

Before propylene glycol can be used in the determination of total water, the presence or absence of a conjugated product must be investigated.

Ethylene glycol. This substance with a molecular weight approximately that of urea has been used by Jacobs and Stewart in quantitative measurements of cell membrane permeability (1932, 1933); by Stewart and Jacobs with relation to temperature changes (1933) and fertilization (1932); also by Jacobs in studies of diffusion processes (1931a, 1931b) and zoological classification (1931c, 1936). On the assumption that glycol is distributed uniformily in "solvent" water of mammalian

erythrocytes and the surrounding medium, Parpart and Shull (1935) and Macleod and Ponder (1936) have used this substance for the determination of solvent water in normal cells. The rapid rate of penetration and the comparative harmlessness of the glycol have shown it to have distinct advantages over other alcohols in such experiments (Jacobs and Stewart). Since it has been used in industry as a preservative for fruit juices, flavors and extracts, as a solvent for drugs, as an antifreeze agent for automobiles and, on occasion, has been consumed in a concentrated form by man, the literature contains many reports on the toxicity of this glycol (Fuller, 1924, Hansen, 1930, Wiley et al., 1936).

Bachem (1917) in pharmacological studies of ethylene glycol observed in dogs that undiluted doses up to 4.1 gm. per Kg. by stomach tube produced no symptoms. However, Page (1927) found that higher doses (5.5 gm. per Kg.) by the same route produced emesis and that intravenous injections above 1 cc. per Kg. quickly lowered blood pressure and accelerated respiration. Von Oettingen and Jirouch (1931) in a comparison of the pharmacological action of this glycol and some derivatives give evidence for the hemolytic action of this compound. Hanzlik et al. (1931) in a more extensive pharmacological investigation noted rapid absorption after intramusular injection as well as after administration by stomach tube or injection into intestinal loops of rabbits and rats. They observed no effect upon smooth muscle in final concentrations of 1:500 or less. Acute toxicity tests in man, rabbit and rat showed

that doses as high as one gm. per Kg. are tolerated. In chronic intoxication there is noted an increase in the oxalate of the urine. This is not apparent in acute experiments with moderate doses, since oxidation probably proceeds to carbon dioxide and water, the final products of glycol metabolism (Hanzlik). Numerous clinical reports refer to ethylene glycol as a poison. The toxic effects have been attributed to the formation of oxalates.

Original observations on ethylene glycol. Studies on the distribution of small doses in the intact animal for the purpose of showing the actual diffusion and oxidation of the substance have not been found in the literature. However, since small intravenous doses might be rapidly distributed in tissues, a study of the quantitative methods of analysis should be considered. The method of Macleod and Ponder (1936) for the analysis of glycol is slow and tedious and the titration end-point of the final reaction is not easily detected. The method described by Parpart (1936) for analysis in aqueous and Ringer's solution was found to be quite accurate in such solutions. Glycol is oxidized by potassium dichromate in the presence of concentrated sulfuric acid to carbon dioxide and water. The dichromate which remains after complete oxidation is titrated electrometrically with ferrous sulfate. The end-point of the latter oxidation-reduction reaction is indicated by a sharp drop in the electrical potential (recorded with a type K potentiometer) of the solution. One drop of the sulfate solution produces a marked change in potential.

The following reactions occur:

$$5K_2Cr_2O_7 + 3HOCH_2 \cdot CH_2OH + 2PH_2SO_4 \xrightarrow{\text{(heat of reaction)}} 6CO_2 + 29H_2O + 5K_2SO_4 + 5Cr_2(SO_4)_3$$

$$K_2Cr_2O_7 + 6FeSO_4 + 8H_2SO_4 \longrightarrow 3Fe_2(SO_4)_3 + 2KHSO_4 + Cr_2(SO_4)_3 + 7H_2O$$

This method is not specific for glycol since the blood and tissues contain substances which are oxidized by these same reagents. Therefore, blanks must be run on the reagents and the tissue samples before the addition of glycol. The results presented in Table IV are those following the preliminary experimentation. They indicate that the method is not sufficiently accurate for the quantitative determination of small changes in the concentration of glycol in blood.

Dimethylurea and Dioxyacetone. In perfusion studies of the cat's submaxillary gland, Amberson and Höber (1933) noted a correlation between the rate of penetration and such properties as lipoid solubility and the molecular size of compounds. The lipoid soluble amides of small molecular size appeared in high concentration in saliva, but those which were lipoid insoluble and larger in molecular size appeared in low concentration or not at all. The lipoid soluble compound, dimethylurea, and dioxyacetone, of small molecular size, penetrate these cells with ease. Experimental studies of the effect of the former substance on the intact animal (mouse) indicate that it belongs to the group of urea

TABLE IV

RECOVERY OF ETHYLENE GLYCOL ADDED TO WATER AND TISSUES

Date	Tissue or Fluid	Substance added in mgm.	Substance recovered in mgm.	Percentage recovery
8/19/36	Water	0.558	0.551	99.0
8/19/36	Water	0.558	0.545	97.7
8/19/36	Water	0.558	0.682	126.0
10/17/36	Water	0.62	0.76	122.0
10/17/36	Water	0.62	0.61	98.4
10/26/36	Water	0.62	0.618	99.6
10/26/36	Water	0.62	0.615	99.3
10/27/36	Water	0.62	0.654	105.0
10/27/36	Water	0.62	0.616	99.36
11/3/36	Water	0.62	0.664	107.0
11/3/36	Blood(dog)	0.31	0.243	79.0
10/17/36	Muscle(g.pig)	1.034	1.13	109.7
10/29/36	Muscle(rabbit)	0.62	0.75	120.9
11/3/37	Muscle(dog)	0.62	0.605	97.6

derivatives which are hypnotic (Buck, Hjort, and DeBeer, 1935). As to the metabolism and fate in the body of dioxyacetone, much evidence shows that it is readily converted into glucose (Ringer and Frankel, 1914, Lusk, 1928, and others) and can be given in the same dose as glucose to relieve insulin hypoglycemia in rabbits (Campbell and Hepburn, 1926). Markowitz and Campbell (1927) have shown in dogs that the liver converts this substance into glucose, but in the absence of the liver, dioxyacetone is distributed generally in the tissues. It is evident from these experiments that studies on perfused or isolated tissues are significant, but they give little insight as to what might be expected to occur in experiments on intact animals.

Thiourea. Thiourea penetrates the fish blood cell very rapidly but the reverse is true for the mammalian blood cell (Jacobs, 1936). It might be stated here that urea itself penetrates the erythrocyte of the fish slowly (722 seconds) and the mammalian erythrocyte rapidly (ox blood cell, 1.5 seconds). Jacobs (1936) reported these observations as characteristic differences between fish and mammalian blood cells. In studies of the hypnotic effects of some thioureas, DeBeer et al. (1936) found this substance to be depressant for mice in doses of 3.0 gm. per Kg. This indicates in a qualitative way the effect of the drug, but does not permit calculation of dosage for higher animals.

Triethylurea. Overton's work (1895, 1896), later discussed and confirmed by Jacobs (1924) and Stewart and Jacobs (1935), shows that a replacement of polar groups by alkyl groups in a molecule permits an increase in the rate of penetration of a substance into plant cells. Overton found that triethylurea enters the cells instantly. Unfortunately, this substance is toxic when given to intact animals (Buck, Hjort and DeBeer, 1935).

Urea. Of all the substances investigated in permeability studies, urea is the one most frequently used and that with which other substances have generally been compared. Studies on isolated cells (Jacobs, 1931a, Jacobs and Stewart, 1932, and Parpart and Shull, 1935, Örskov, 1935, Macleod and Ponder, 1936) and on the distribution of urea in the tissues of the animal body have been reported by many investigators. The data obtained by Marshall and Davis (1914), Gad-Andresen (1919), and others are tabulated on the following pages in order to organize some of the extensive material on this subject. Table V does not include results of analyses for each tissue, since these are frequently different for different workers. This can be attributed to the various methods of analysis used. It should be stated, however, that the results obtained by a single analyst generally indicate equal distribution of urea. The investigators listed in Table V are those who for the most part have been concerned primarily with the distribution of urea, a fact which is apparent from the titles of their reports. That animal

TABLE V

METHODS OF ANALYSIS OF TISSUES FOR UREA

Date	Investigator	Method of Analysis	Animal	Tissues Analyzed
1905	Benedict	Kjeldahl	man	sweat
1910	Macallum	Folin and Wu (nesslerization)	dogfish	blood
1914	Denis	Folin and Denis	fish	blood
1914	Marshall and Davis	Marshall urease aeration	dog	all tissues
1915	Woods	Van Slyke and Cullen (urease aeration)	man	blood and spinal fluid
1915	Cullen and Ellis	Van Slyke and Cullen	man	blood and spinal fluid
1916	Sumner	Van Slyke and Cullen	cat	blood and muscle
1919, 1922	Gad-Andresen	Van Slyke and Cullen	frog rat dog	all tissues and fluids, confirms Marshall and Davis
1919	Myers and Fine	Van Slyke and Cullen, Folin	man	blood and spinal fluid
1920	Benson	Sumner urease aeration	dogfish	muscle
1922	Wu	Folin and Wu	man	corpuscles and plasma

TABLE V (continued)

1922	Denis, Sisson and Aldrich	Folin and Wu, Denis and Minot	goats	blood and milk
1922	Denis	Folin and Wu	fish	blood
1922	Hench and Aldrich	Van Slyke and Cullen	man	saliva
1925	Calvin and Isaacs	Hench and Aldrich (mercury compound)	man	saliva
1927	Duke-Elder	Urease method (?)	horse	blood and aqueous humor
1927	Rietti	Van Slyke and Cullen	dog	body fluids
1928	Kiech and Luck	Xanthydrol method	rat (?)	whole animal
1930	Eggleton, P.	Kjeldahl	frog	muscle
1933	Walker	Van Slyke and Cullen Xanthydrol method	frog rabbit dog man	fluids: aqueous humor, cerebrospinal, lymph, blood
1934	Root	Van Slyke and Cullen	dogfish	nerve
1934	Peskett	Van Slyke and Cape	cow	milk and blood
1934	Conway and Kane	Aeration method (Van Slyke & Cullen ?)	frog	blood and muscle
1935	Parpart and Shull	Hypobromite	man beef dog rabbit sheep	red blood cells

TABLE V (continued)

1937	Graham et al.	Van Slyke manometric	goat	blood
1937	Benham	Conductometric method of Ranganathan and Sastri	cat dog rabbit	blood and aqueous humor

tissues in general are permeable to urea is well established by these results.

In man the estimation of urea in the different body fluids has been studied for the purpose of selecting some source other than blood for an index of urea retention in kidney disease. Body fluids (except urine) show approximately the same concentration as the blood; the urea in saliva, for example, gives a clear prognosis of nitrogen retention (Hench and Aldrich, 1922, Schmitz, 1923, and Calvin and Isaacs, 1925).

Since urea passes freely from the blood to the gut and vice versa (Pendleton and West, 1932), and is equally distributed throughout all the media of the body (Peters, 1935), it deserves careful investigation as a possible substance for the determination of total body water. In a consideration of the pharmacology and the related effects of urea, Macht (1914) has found small doses ineffective in retarding activity of rats. However, urea was reported by Grehant and Quinquaud (1884) to be poisonous to the dog in doses which raise the blood level to 0.6 gm. per 100 cc. Their results agree with those of Herter and Wakeman (1899) and Hewlett et al. (1916) on man and dog. Hewlett and his co-workers found that for every gram of excess urea in the body of man there was an increase in the blood concentration of 2.5 mgm. per 100 cc. Up to 90 mgm. per 100 cc. there were no symptoms; but when a maximum concentration of 150 to 160 mgm. was reached, there were marked gastro-intestinal disturbances, headache, fatigue and unsteady hands, but no marked change in blood pressure.

If the blood level continues to rise, prostration, apathy and somnolence occur. This is to be expected for, according to DeBeer et al. (1935), one-half to two-thirds the lethal dose is hypnotic.

Marshall and Davis have found that the dog can rid its body of injected urea in 24 hours, provided a favorable state of water balance is maintained. These investigators find no evidence of transformation of urea into other substances in the body. McCaskey (1914) found in man that after ingestion of 30 gm. urea, 15-16 per cent was excreted in 2-3 hours and 60-70 per cent lost in 12 hours. However, the rate of excretion is dependent upon the state of water balance and the quantity of urea given (McLean and Selling, 1914, Addis and co-workers, 1917a, 1917b, 1918a, 1918b, 1923a, 1923c, Taylor et al., 1923, Drury, 1923, Bourquin, 1924, 1925, Kay and Sheehan, 1933, Dominguez, 1935, Kaplan and Smith, 1935). If these factors are controlled, then urea should give a fairly accurate index of the water content of the body.

There are several methods for the quantitative determination of urea in small samples of tissues and fluids. The most accurate of these are the volumetric or manometric gasometric urease method of Van Slyke (1917, 1927), the aeration-titration method of Van Slyke and Cullen (1914), the aeration-titration method of Kay and Sheehan (1934) and the conductometric method of Ranganathan and Sastri (1936). Some methods involve the decomposition of urea by heat or its digestion by urease with the production of ammonium

carbonate (Peters and Van Slyke, Methods, 1932, p. 546). From the latter molecule either ammonia or carbon dioxide can be liberated and determined by aeration-titration or gasometric methods. Other procedures involve the conjugation of urea with another compound, such as the formation of complex molecules with mercury or xanthydrol, or the liberation of the nitrogen in the urea molecule.

Original observations. The results obtained with the methods that have been tested by the author are tabulated in Table VI. It will be noted that the percentage error with the aeration-titration method is considerable. Incomplete aeration and detection of color change in the titration were believed to be responsible for this. It was found that aeration for 20-25 minutes instead of 15 minutes (the time interval for the previous analyses) was required for extraction of the ammonia present. This reduced the error to 0.6-3.0 per cent, which is still a wide range. In the titration procedure, the large quantity of dilute solution resulting from titration is sufficient to prevent a sharp color change. But a large quantity of dilute acid must be present in the aeration tube to dissolve the slowly extracted ammonia. It has been found that one drop of the dilute alkali will cause the error to fluctuate 0.8 per cent, accounting for the wide range noted above. Such errors do not occur in the gasometric methods.

From a comparison of the duplicate analyses in Tables VII and VIII, it appears that the most accurate results

TABLE VI

RECOVERY OF EXOGENOUS UREA

VAN SLYKE MANOMETRIC GASOMETRIC METHOD

Date 1936	Fluid	Endogenous Content Mgm. %	Mgm. Urea added to 100 cc.	Urea Theoretical Mgm. %	Urea Recovered Mgm. %	% Error
6/5	water	0	15	15	15.4	+ 2.6
6/5	water	0	30	30	28.54	- 4.8
6/5	water	0	60	60	59.26	- 1.2
6/6	water	0	15	15	15.4	+ 2.6
6/6	water	0	30	30	30.4	+ 1.3
6/8	urine	568.0	15	299.1	305.9	+ 2.3
6/8	urine	568.0	30	314.1	312.5	- 0.5
6/9	urine	450.1	15	240.1	227.2	- 5.2
6/9	urine	450.1	30	255.1	245.2	- 3.9
6/10	urine	315.5	15	165.2	165.8	+ 0.1
					average	2.43

RECOVERY OF EXOGENOUS UREA

VAN SLYKE AND CULLEN AERATION-TITRATION METHOD

(Samples aerated for 15 minutes)

7/8	water	0	60	60	55.2	- 8.0
7/9	water	0	60	60	62.4	+ 4.0
7/10	urine	1993.5	60	1026.7	1126.8	+ 9.72
7/10	urine	1187.4	60	623.7	614.4	- 1.5
7/12	water	0	60	60	59.4	- 1.0
7/12	urine	1847.8	60	953.6	993.8	+ 4.4
7/13	urine	662.4	60	361.7	328.8	- 9.0
7/14	water	0	60	60	62.4	+ 4.0
7/14	urine	256.7	60	158.3	149.4	- 5.6
7/17	saliva	27.6	60	43.8	39.6	- 9.6
7/18	water	0	60	60	58.0	- 3.3
7/18	saliva	27.5	60	43.7	40.8	- 7.2
					average	5.61

(Samples aerated for 25 minutes)

7/20	water	0	60	60	58.3	- 2.8
7/21	water	0	60	60	60.4	+ 0.66
7/21	water	0	60	60	61.9	+ 3.1
7/21	water	0	60	60	61.8	+ 3.0
					average	2.39

TABLE VII

DUPLICATE ANALYSES

VAN SLYKE AND CULLEN AERATION-TITRATION METHOD

(Samples aerated for 15 minutes)

Date	Tissue or Fluid	Analyses Mgm. per cent		Percentage Error
7/10/36	Urine(man)	1187.4	1274.1	6.75
7/13/36	Urine(man)	662.4	673.0	1.57
7/15/36	Saliva(man)	28.8	28.7	0.35
7/17/36	Saliva(man)	28.1	27.6	1.78
7/17/36	Blood(dogfish)	1741.0	1788.0	2.64
7/18/36	Blood(dogfish)	1868.0	1921.0	2.76
7/18/36	Blood(dogfish)	1815.0	1863.0	2.58
			average -	2.63

(Samples aerated for 25 minutes)

8/1/36	Blood(dogfish)	2016	1995	1.0
8/5/36	Blood(dogfish)	1927	1884	4.45
8/6/36	Blood(dogfish)	1965	1905	3.06
8/13/36	Blood(dogfish)	1768	1763	0.28
8/14/36	Blood(dogfish)	1736	1707	1.67
8/4/36	Muscle(dogfish)	1580.6	1519.4	3.86
8/6/36	Muscle(dogfish)	1281.9	1291.4	0.73
8/14/36	Muscle(dogfish)	1136.6	1109.6	2.37
			average -	2.08

TABLE VIII

DUPLICATE ANALYSES

VAN SLYKE MANOMETRIC GASOMETRIC METHOD

Date	Tissue or Fluid	Analyses Mgm. per cent		Percentage Error
6/8/36	Urine(man)	568.0	575.96	1.3
6/9/36	Blood(man)	22.7	22.8	0.43
6/11/36	Blood(man)	24.2	23.6	2.48
			average -	1.40

DUPLICATE ANALYSES

VAN SLYKE VOLUMETRIC GASOMETRIC METHOD

7/30/36	Blood(dogfish)	2072.0	2018.0	2.6
7/31/36	Blood(dogfish)	1936	1963	1.4
8/3/36	Muscle(dogfish)	2192.4	2269.5	3.4
8/4/36	Muscle(dogfish)	1155.3	1160.3	0.4
8/5/36	Muscle(dogfish)	1607.5	1643.9	2.1
8/13/36	Muscle(dogfish)	1405.25	1376	2.06
8/14/36	Muscle(dogfish)	1385.54	1376.78	0.64
8/15/36	Muscle(dogfish)	1299.4	1268	2.38
8/13/36	Blood(dogfish)	1968	1987	0.95
8/14/36	Blood(dogfish)	2019	2039	1.0
8/31/36	Blood(dogfish)	1731	1704	1.56
9/1/36	Blood(dogfish)	1786	1854	3.2
			average -	1.81

are obtained by the gasometric method. This method, therefore, was selected for experimental work where changes in urea of the order of 5 to 10 mgm. were to be detected in 35 to 85 mgm. per cent solutions.

In a study of 10 urea methods, Seljeskog and Cavett (1937) also found that results for the manometric gasometric method showed the least maximal variation. However, the aeration-titration as well as the gasometric method yielded accurate results for ranges of 20 to 120 mgm. per cent urea solutions.

Preliminary experiments (Table IX) were conducted in order to compare the water and urea in the blood and in the rectus abdominis muscle of four anesthetized animals. Although the results are variable, due to imperfections in the technic of preparation and drying of samples (corrected in later experiments), they indicate that the water content of the muscle can be predicted from its urea concentration, when the water and urea levels of the blood are known.

TABLE IX

COMPARISON OF WATER AND UREA CONTENT IN TISSUES

Animal	Tissue urea Mgm./100 Gm.		Water content of tissue* Gm./100 Gm.		
	Blood	Muscle	Blood	Muscle	
			Actual	Actual	Calculated**
G. pig	27.2	22.9	82.5	80.0	68.8
Rabbit	27.9	25.9	81.0	75.8	75.2
Dog	22.98	23.7	80.0	77.3	82.4
Dog	19.47	22.2	79.5	76.4	90.6

*Tissues were dried in oven at 110 C.
**Calculations were based on urea analyses of both tissues and the water content of the blood.

Sulfanilamide*. Although no permeability studies of para-amino-benzene-sulfonamide have been found in the literature there seems to be no doubt that this substance diffuses throughout all the tissues of the body.

Most of the literature on sulfanilamide deals with: (1) the bacteriostatic action of the drug, and its related compounds in vitro and in vivo (Colebrook et al., 1936, Fuller, 1937, Long and Bliss, 1937, Finklestone-Sayliss et al., 1937, Mellon et al., 1937, Rosenthal, 1937a, 1937b, Osgood, 1938, and others); (2) its therapeutic value (Veil, 1934, Domagk, 1935, Schreus, 1935, Hörlein, 1935, Trefouël et al., 1935, Levaditi and Vaisman, 1935, Anselm, 1935, Buttle et al., 1936, Scheurer, 1936, Colebrook and Kenny, 1936, Proom, 1937, Schwentker et al., 1937, Walther, 1937, Helmholz, 1937, Dees and Colston, 1937, Bohlman, 1937, Branham and Rosenthal, 1937, Rosenthal et al., 1937, and others); and (3) the toxic effects after prolonged administration (Discombe, 1937, Kohn, 1937, Newman, 1937, Frank, 1937, Bucy, 1937, Harvey and Janeway, 1937, Bigler et al., 1938, Marshall and Walzl, 1937, Marshall et al., 1938, and others).

The first report on "Prontosil" by Schreus (1935) indicated that pharmacologically this drug, related to sulfanilamide, was extraordinarily inert no matter how it was given (subcutaneous, intravenous or oral). Yet, in infection, all methods were effective therapeutically. He observed that

*Name officially adopted by the Council on Pharmacy and Chemistry of the American Medical Association for para-amino-benzene-sulfonamide.

the fatal intravenous dose for the mouse was 0.75 to 1.0 gm. per Kg.

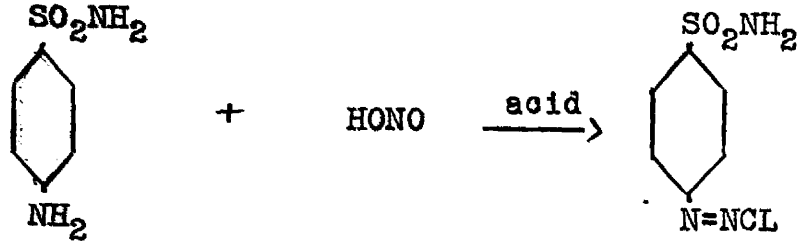
Weese and Hecht (1935), in the research laboratory of the I. G. Farbenindustrie, Elberfeld, Germany, where "Prontosil" was synthesized, found: (1) no change in blood pressure or heart activity of cats and rabbits after intravenous injections of 10 mgm. per Kg.; (2) no effect on smooth muscle of the uterus or intestine; (3) no symptoms in animals after subcutaneous injections of 1 gm. per Kg.

Apparently this drug is pharmacologically inert in small doses and is distributed rapidly and uniformily in the tissues, possibly in 60 to 80 per cent of the body weight (Marshall et al., 1937a, 1937c). More recently Marshall et al. (1938) have reviewed and re-investigated the pharmacology of sulfanilamide and its derivatives. They conclude that sulfanilamide is not toxic in moderate doses and that prolonged administration produces no permanent injuries to the tissues.

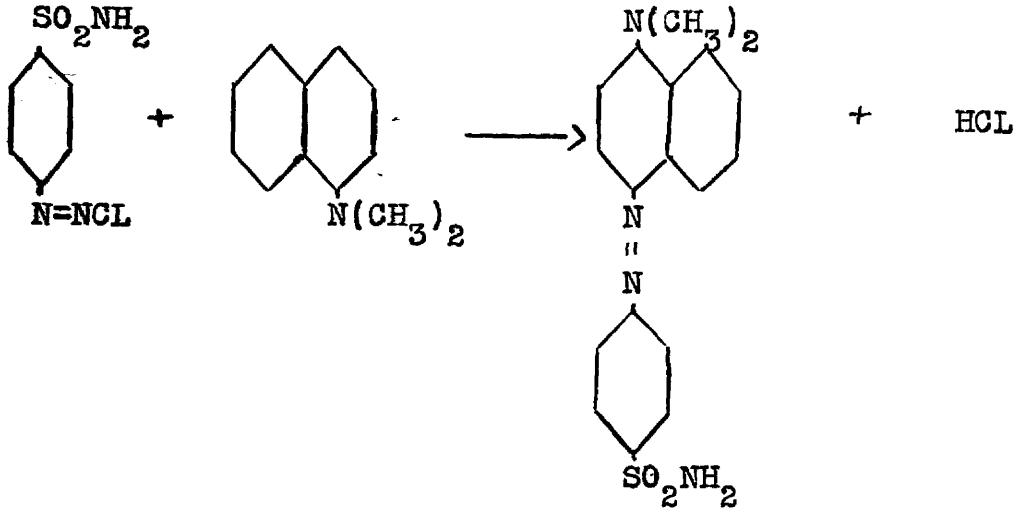
Original observations. A spectrophotometric modification of Marshall's method of analysis (1937a, 1937b and Marshall et al., 1937a) was developed by the author in order to: (1) eliminate the preparation of standards, (2) permit analysis of 0.5 cc. samples, and (3) increase accuracy.

The blood samples were hemolyzed with 7 parts of water; proteins were removed with 2 parts of 20 per cent trichloracetic acid. Ten parts of the filtrate were treated with one part of 0.1 per cent sodium nitrite and five parts of 0.4 per cent alcoholic dimethyl-α-naphthylamine to obtain the

red azo dye. The reactions are as follows:



Sulfanilamide



red azo dye (1- dimethyl amino-4, azo benzene, p-sulfonamide naphthalene)

The dye obtained on coupling by Marshall's method shows absorption in the visual spectrum in the range of 430 mμ to 600 mμ with maximal absorption at 530 mμ* (see Fig. 1).

Since the drug is conjugated in rabbit and man (Marshall, Cutting and Emerson, 1937), and the determination of body

*Allport (1936) found that his colored compound had "approximate maximal absorption" at 480-525 mμ. He was primarily interested in qualitative tests for the detection of impurities, such as sulfanilic acid which shows "approximate maximal absorption" at 520-530 mμ.

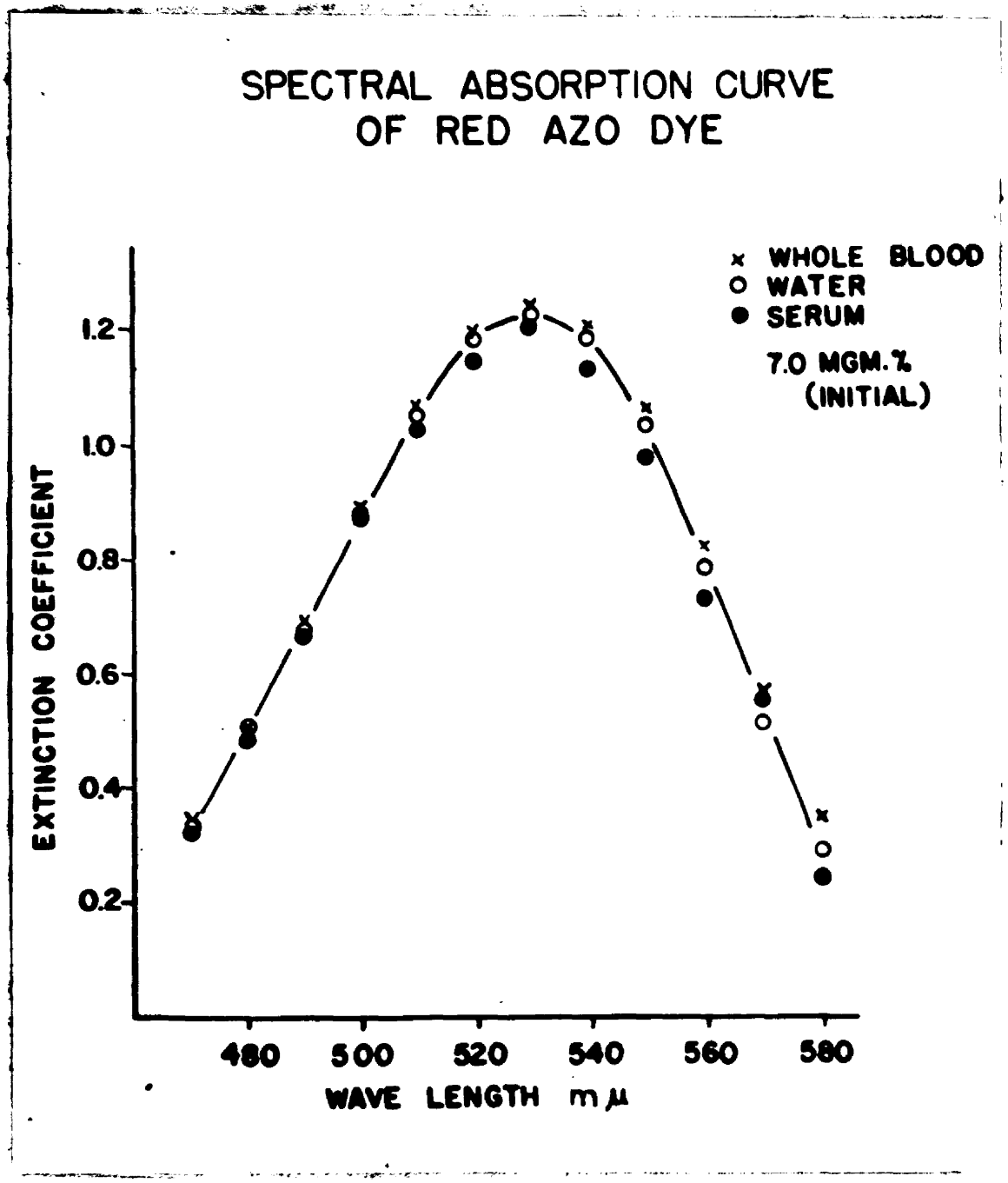


Fig. 1. Curve showing comparable results in the region of 480 to 580 m with maximal absorption in each analysis at 530 m . The concentration of sulfanilamide solution analyzed falls in the range of blood levels in a typical experiment.

fluids requires acid hydrolysis with heat, Marshall (1937b) has suggested the use of p-toluene sulfonic acid for the precipitation of proteins. A comparison was made of the results with this precipitant and with trichloracetic acid. Fig. 2 shows that there is complete recovery of sulfanilamide in both cases. In experiments on dogs, in which sulfanilamide is not conjugated, trichloracetic acid has been used in the analyses throughout the present investigation.

Fuller's method (1937), in which sulfanilamide is diazotized and then coupled in alkaline solution with a phenol, yields a yellow dye. With increased concentration this color changes from orange to a deep red. Fuller does not give the results of "in vitro" experiments, but Marshall (1937b) has indicated that the yields with this method are low. Since there are definite changes in color, several peaks in the absorption spectrum of the dye would probably occur in a wide range of concentrations.

Absorption curves of sulfanilamide diazotized and coupled in water solutions, in serum and in whole blood appear in Fig. 1. The original concentration of sulfanilamide was 7.0 mgm. per cent. These results agree exactly with those obtained for 5 and 8 mgm. per cent solutions. In the analysis of the substance in concentrations from 0 to 15 mgm. per cent, it was found that the optical density or extinction coefficient at 530 $m\mu$ is directly proportional to the concentration of sulfanilamide (Beer's law) (see Fig. 3). The effect of any phenolic compounds or other substances in the fluids

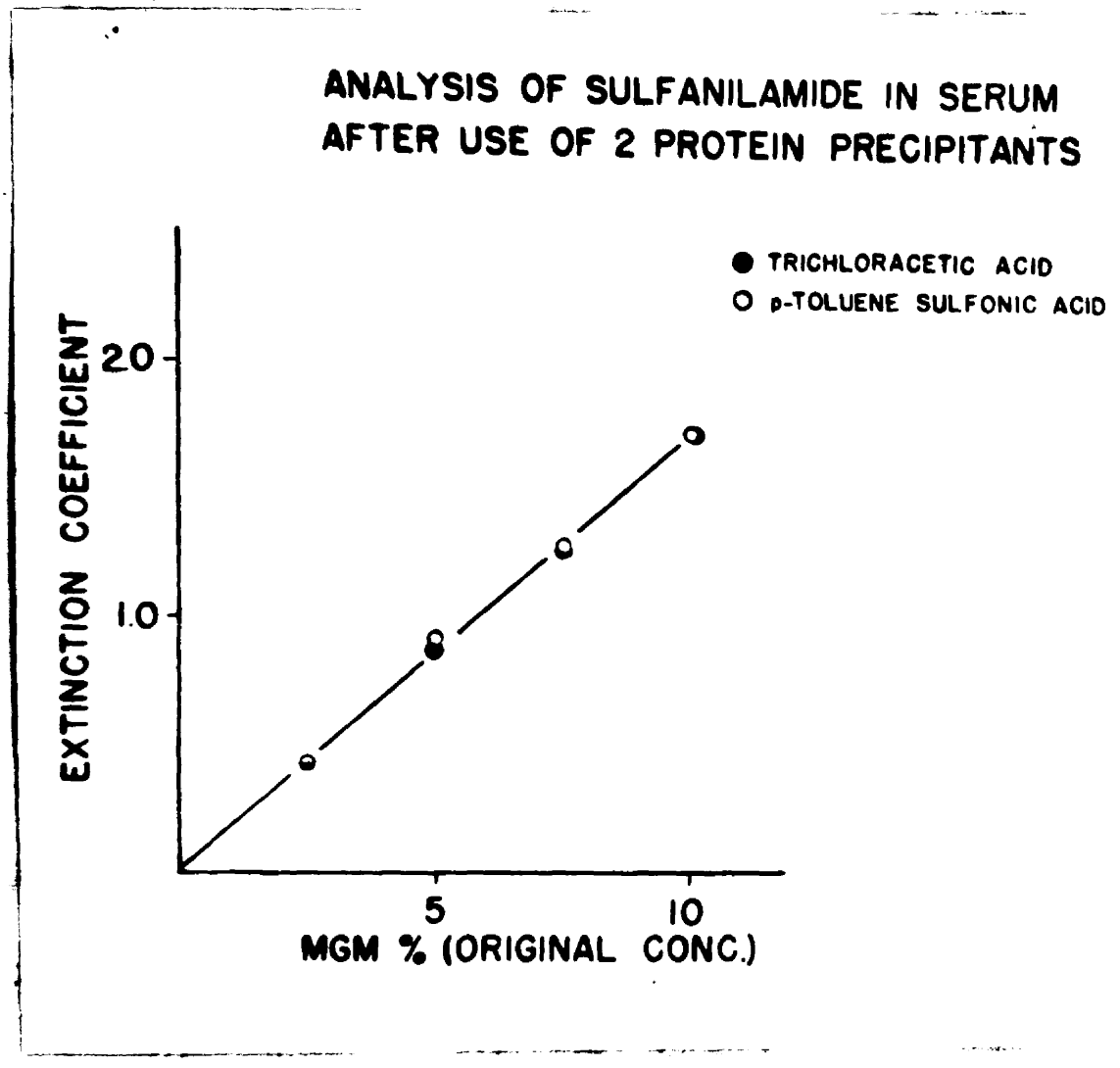


Fig. 2. Showing the complete recovery of the sulfanilamide in the form of the red azo dye after the proteins of the blood have been precipitated in separate experiments by trichloracetic and p-toluene sulfonic acids.

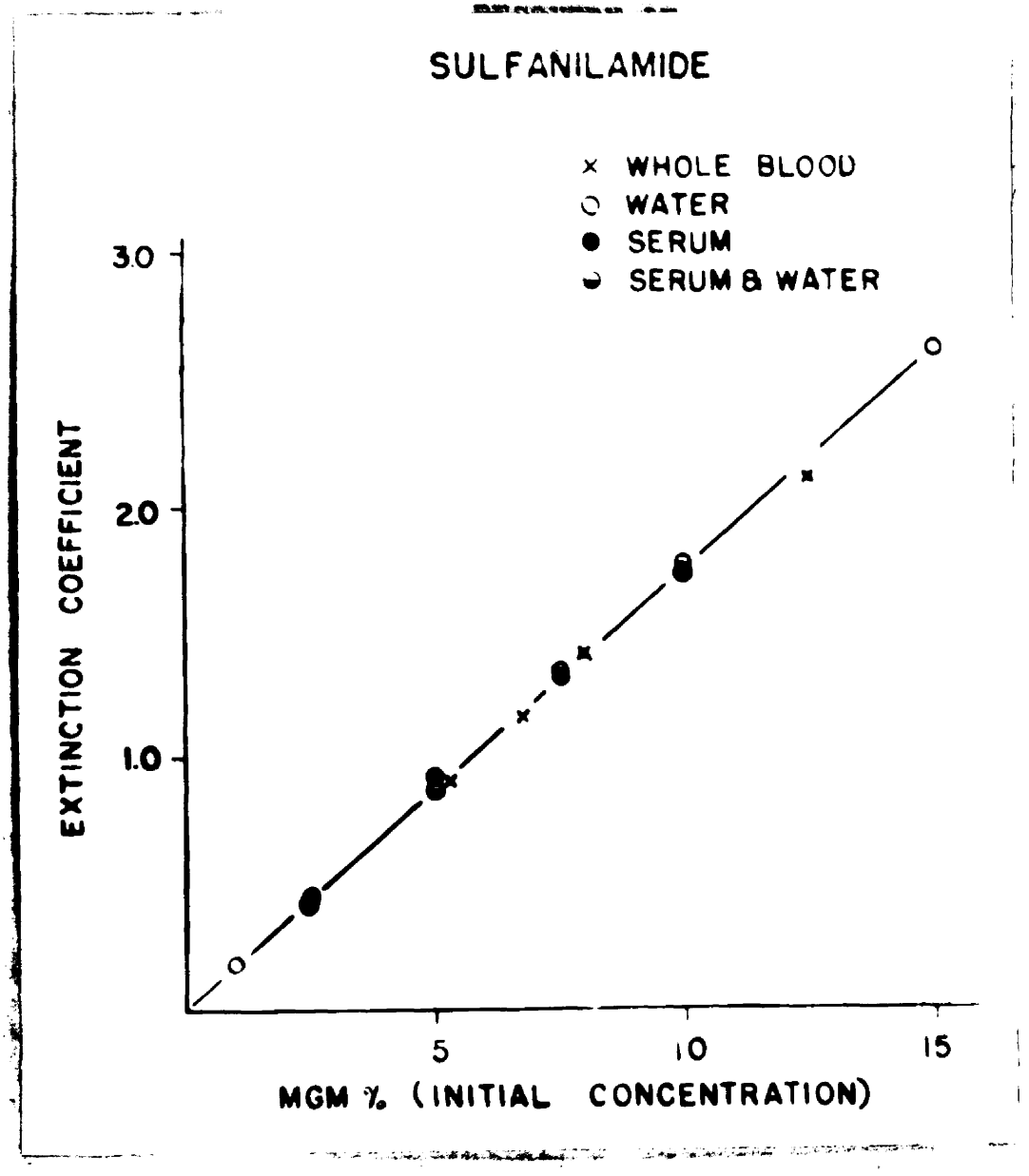


Fig. 3. Curve showing complete recovery of sulfanilamide, as the red azo dye, from serum and whole blood after precipitation of proteins by trichloracetic acid. Such a curve follows Beer's law and indicates that the factor, 5.7, will convert extinction coefficient into mgm. per cent.

which may react with the reagents used for the diazotization is cancelled by reading the unknown in a Königs-Marten spectrophotometer against an appropriate blank.

The accuracy of this sulfanilamide method was tested by a series of duplicate analyses and recovery experiments in which sulfanilamide was added to water, serum and whole blood. Tables X and XI show the results of such experiments and indicate the maximal error to be 2.0 per cent, with an average error of 0.8 per cent for 18 duplicate analyses, and 1.07 per cent for 11 recovery analyses.

A preliminary experiment was performed to determine the distribution of sulfanilamide in cells and serum in relation to their water content. One hundred cc. of a warm solution containing 800 mgm. sulfanilamide per 100 cc. of 0.5 per cent sodium chloride was injected slowly into the left jugular vein of a dog. After 2 hours, blood samples were withdrawn. Some were collected under oil, others in heparin and oxalate. The analysis of serum and whole blood did not show a direct relation to water content. Investigations were carried further in order to determine to what the difference might be attributed. The final results showed that some sulfanilamide was distributed in the oil as well as the serum and that the red blood cells as they clotted were trapping and holding some of the substance, probably in combination with or adsorption on the hemoglobin. This difference in content in serum and whole blood was not discernible in the previous analyses, because the sulfanilamide had been added directly

TABLE X

DUPLICATE ANALYSES OF SULFANILAMIDE

IN WATER AND TISSUE FLUIDS*

Sample	Values Expressed in Ext. Coeff.		Percentage Error
Water	0.863	0.852	1.0
Water	1.798	1.769	1.5
Water	0.864	0.863	0.1
Water	1.439	1.447	0.5
Blood Serum(dog)	1.750	1.740	0.5
Blood Serum(dog)	1.786	1.750	2.0
Blood Serum(dog)	1.265	1.269	0.3
Blood Serum(dog)	1.603	1.634	2.0
Blood Serum(dog)	1.295	1.285	0.8
Blood Serum(dog)	1.735	1.728	0.4
Blood Serum(dog)	0.432	0.433	0.2
Whole Blood(human)	0.659	0.671	1.8
Whole Blood(dog)	1.431	1.429	0.1
Whole Blood(dog)	1.265	1.263	0.1
Whole Blood(dog)	1.163	1.141	1.0
Whole Blood(dog)	1.005	0.997	0.8
Spinal Fluid(human	1.563	1.575	0.8
Spinal Fluid(human)	0.818	0.823	0.6
		Average	0.81

*For coupling procedure 1:10 dilution was used.

TABLE XI

RECOVERY OF SULFANILAMIDE ADDED TO WATER AND TISSUE FLUIDS*

Sample	Theoretical in mgm. %	Actual mgm. %	Percentage Recovery	Deviation from 100%
Water	5.0	5.1	102.0	2.0
Water	10.0	10.1	101.0	1.0
Water	15.0	14.9	99.6	0.4
Water	2.5	2.4	96.8	3.2
Water	1.0	1.0	100.0	0.0
Water	4.0	4.1	101.6	1.6
Serum (dog)	5.0	4.9	98.0	2.0
Serum (dog)	7.5	7.5	100.0	0.0
Serum (dog)	10.0	10.1	101.0	1.0
Serum (dog)	2.5	2.5	98.0	2.0
Serum (dog)	5.0	5.1	102.0	2.0
Serum (dog)	7.5	7.4	98.6	1.4
Serum (dog)	10.0	9.9	99.0	1.0
Serum (dog)	2.5	2.5	100.0	0.0
Serum (dog)	5.0	5.0	100.0	0.0
Serum (dog)	7.5	7.4	98.6	1.4
Serum (dog)	10.0	9.9	99.0	1.0
Whole blood (dog)	5.2	5.1	97.9	2.1
Whole blood (dog)	6.9	6.9	100.3	0.3
Whole blood (dog)	8.0	8.1	100.8	0.8
Whole blood (dog)	12.5	11.9	96.0	4.0
			Average	1.3

*For coupling procedure 1:10 dilution was used

to the serum (from which the clot had previously been removed), to oxalated whole blood and to water. In the whole blood analysis, the process of hemolysis releases the trapped substance, an indication that sulfanilamide is not in combination with the constituents of the cell membrane.

Marshall has not mentioned this difference between serum and whole blood in his numerous publications, but has continued to use whole blood for all analyses, since his data (personal communication) indicated that on the basis of water content whole blood analysis gave approximately the same concentration of sulfanilamide as other body tissues. Analyses in the present investigation have also been made on whole blood samples. However, the distribution between cells and plasma should be investigated further.

CHAPTER V

DETERMINATION OF THE WATER AVAILABLE FOR DILUTION OF UREA AND SULFANILAMIDE IN UNANESTHETIZED DOGS

It has been pointed out in Chapter IV that urea and sulfanilamide appear to be distributed in all the tissues according to their water content. Hence it should be possible to determine total body water by measuring the extent to which a known amount of either of these substances, injected intravenously, is diluted in the body. In carrying out the determination one must of course know the time required for uniform distribution and also how much has been lost from the body during the period of "mixing". This information may be obtained from a careful study of the time-concentration curve of the substance in the blood stream. During the first hour after intravenous injection of one or the other of the se substances, its concentration in the blood falls very rapidly, but subsequently the rate of disappearance remains approximately constant for several hours. The point at which the time-concentration curve approaches a "straight line" presumably signifies the time when the substance is uniformily distributed. The slope of the "straight" part of the curve is then a measure of the rate at which the substance is excreted. On the assumption that the rate of excretion is the same during the "mixing" period as in the later part of the experiment, one may determine the concentration, C_o, which would be obtained if distribution were complete and none of

the substance were lost from the body, by extrapolating the straight portion of the time-concentration curve back to the time of injection. If C_o is expressed in mgm. per kilogram of water, and Q equals the mgm. of urea or sulfanilamide injected, the total body water in kilograms is equal to

$$\frac{Q}{C_o} \qquad (1)$$

If the substance in question is excreted only by the kidney the total available water may also be calculated from the formula

$$\frac{Q - Q_e}{C_t} \qquad (2)$$

in which C_t is the blood concentration at any time after distribution is complete the substance is uniformily distributed and Q_e the amount excreted in the urine during the same interval. If the values for total available water, calculated by formulae 1 and 2, agree one must conclude that the extrapolation method of correcting for disappearance during the mixing period is valid. The evidence presented lends strong support to this conclusion and, furthermore, indicates that the water available for dilution of urea and sulfanilamide is equal to total body water.

The dogs used in the following experiments were carefully selected as to age (1½ to 2 years), type and size (11 to 14 Kg.), since both age and the amount of body fat are known to influence per cent total body water (see Chapter II). All the animals were deprived of food for 16 hours

prior to the experiment, but they were given water "ad libitum". The dogs were trained to lie quietly on the table, so that blood samples* could be withdrawn from and the solutions injected into the right or left external jugular vein without the use of anesthetics.

Water available for dilution of urea in normal dogs

The results of a series of control experiments showing the constancy of the endogenous urea blood levels of five animals are presented in Fig. 4. The concentrations did not vary more than 1.5 per cent. However, when the analyses were done in random order the concentrations appeared to vary 4-6 per cent (see "x", Fig. 4). For this reason, the samples were always kept in the ice-box at a temperature of 12-15 C. until the end of the experiment when they were analyzed in the order in which they were taken.

From a study of the time-concentration curves of urea in the blood, when three different doses (approximately 50, 85 and 350 mgm. per Kg.) are administered** it appears that in each case equilibrium between blood and tissues is approached about an hour after injection (see Fig. 5). Table XII gives the values for disappearance from blood in per cent per hour for each of these doses. The results show that disappearance from blood is proportional to concentration

*The samples were collected under oil. Serum was recovered and analyzed for urea by the volumetric gasometric method of Van Slyke and Stadie. Solid content of the serum was determined by the refractometer.

**Ten per cent urea in 0.5 per cent sodium chloride solution was used for injection.

UREA CONCENTRATION IN THE BLOOD SERUM
OF NORMAL DOGS

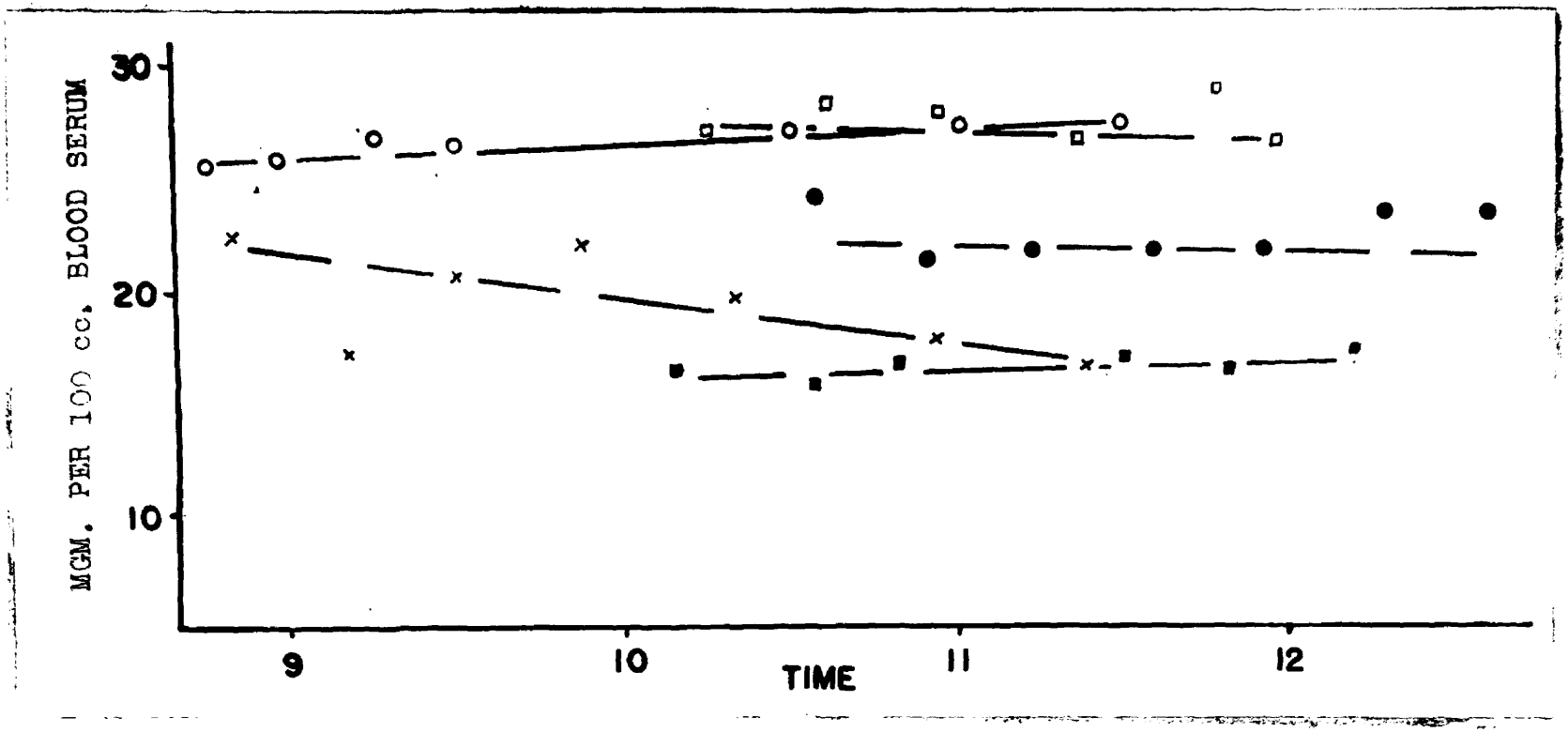


Fig. 4. Curves showing the constancy of serum levels of endogenous urea in 5 different animals. The analyses were performed in the order in which the blood samples were withdrawn, except in one experiment which shows quite variable results. In this particular experiment, represented by "x", the analyses were done in random order.

DISAPPEARANCE OF UREA FROM THE BLOOD
(following 3 different doses)

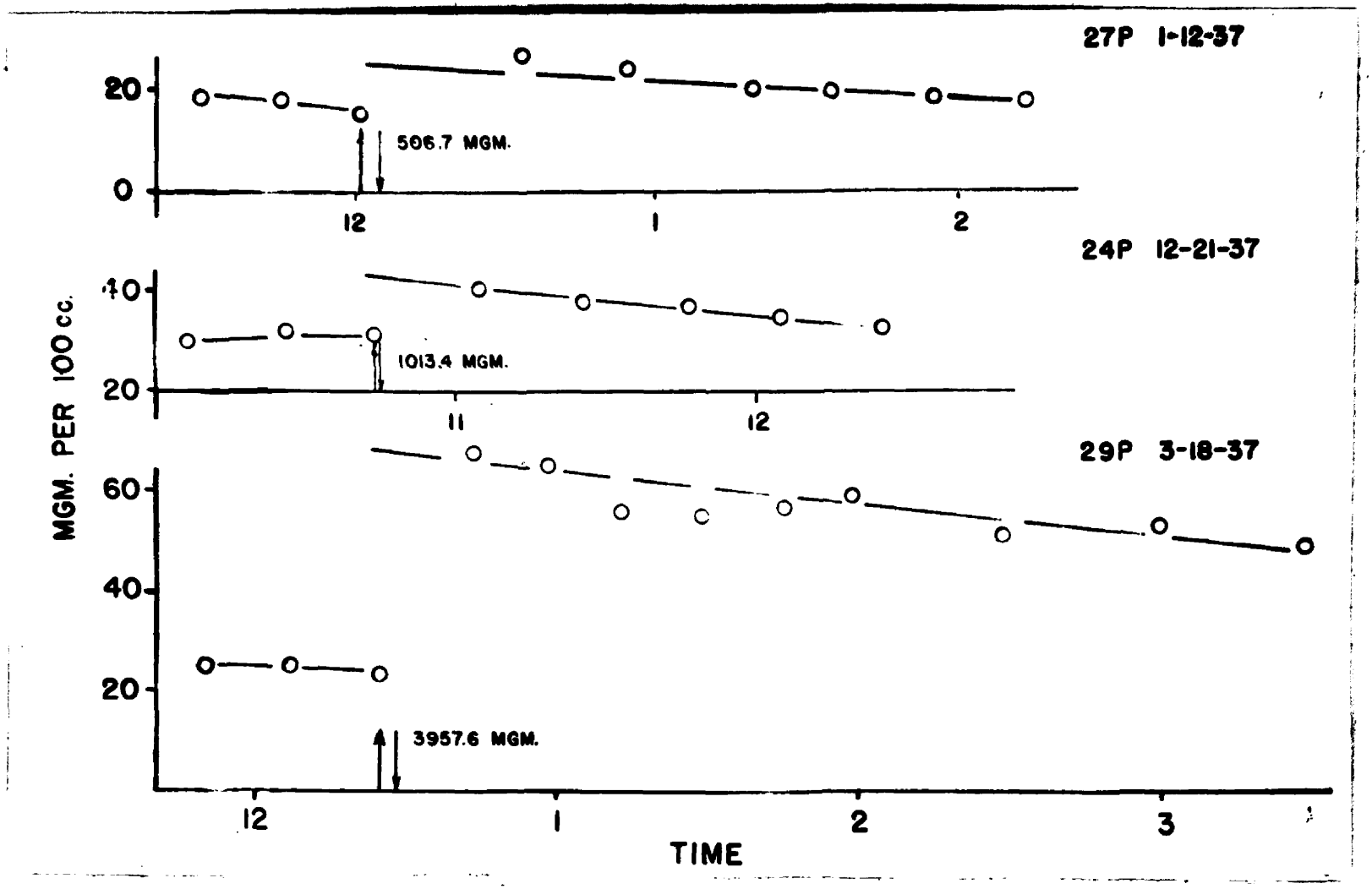


Fig. 5. Three curves showing the original endogenous urea levels and the urea levels after intravenous injection. The "straight" portion of the curves, attributed to excretion, has been extrapolated back to the time of injection.

TABLE XII

WATER AVAILABLE FOR DILUTION OF UREA

Date	Dog	Weight in Kg.	Amount Injected Total Mgm.	Amount Injected Mgm./Kg.	Water in Serum Gm./100 Gm.	Disappearance rate % per hr.	Total water liters	Water % B.W.
1/5/37	23P	13.3	506.7	38.05	94.2	2.9	8.67	65.2
1/7/37	24P	11.2	506.7	45.2	94.5	13.0	6.945	61.9
1/9/37	20P	8.81	506.7	57.4	94.4 (?)	15.2	5.28	60.0
1/12/37	27P	8.57	506.7	58.4	94.4 (?)	13.5	5.26	61.5
12/21/36	24P	11.8	1013.4	86.0	94.5 (?)	15.0	7.99	67.6
2/13/37	24P	11.52	1013.4	88.0	94.6	15.0	6.91	60.2
1/25/37	29P	14.5	1013.4	70.0	94.0	14.0	--	--
1/30/37	25P	11.36	1013.4	89.2	94.4	13.5	--	--
3/13/37	24P	11.33	3957.6	348.3	94.26	9.0	6.94	61.2
3/31/37	24P	11.29	3957.6	350.5	95.0	9.25	7.68	68.0
4/19/37	24P	11.5	3957.6	344.0	94.4	9.37	6.97	60.6
3/18/37	29P	13.86	3957.6	285.0	94.0	10.3	8.60	62.0
4/4/37	29P	14.36	3957.6	275.5	94.33	15.0	8.92	62.1
3/20/37	21P	10.5	3957.6	376.0	94.8	10.7	6.73	64.0
3/25/37	23P	11.9	3760.3	316.0	93.9	10.3	--	--

when blood levels range from 25 to 75 mgm. per cent for an animal in "normal water balance" with no marked diuresis. Reports in the literature by Addis and co-workers (1917a, 1917b, 1918, 1918a, 1918b, 1923a, 1923b, 1923c), Drury (1923), Bourquin (1924, 1925), Kay and Sheehan (1933), Van Slyke et al. (1934), Kaplan and Smith (1935) and Dominquez (1935), indicate that there is not complete agreement about the factors controlling the disappearance of urea from the blood and its excretion in the urine.

In calculating the water available for dilution of urea by formula 1 one must correct C_o for the endogenous urea level determined prior to the injection.

The results (Table XII) range from 60 to 68 per cent of the body weight, with an average of 62.9 for 12 experiments. Duplicate results were obtained for two dogs over a period of 3-4 months during which time the animals' weights were approximately constant.

If these results are compared with those from the complete protocols of Marshall and Davis (1914) for blood urea concentration (2-3 day period) after large single doses, (see Fig. 6), it appears that a 3-4 hour period is sufficiently long to obtain adequate data. It is interesting to note that by calculating Marshall's data according to the formula presenting previously, one obtains the figure 66.5 per cent body for the water content of a dog weighing 6.8 Kg. and 69.4 per cent for a dog weighing 13.6 Kg.

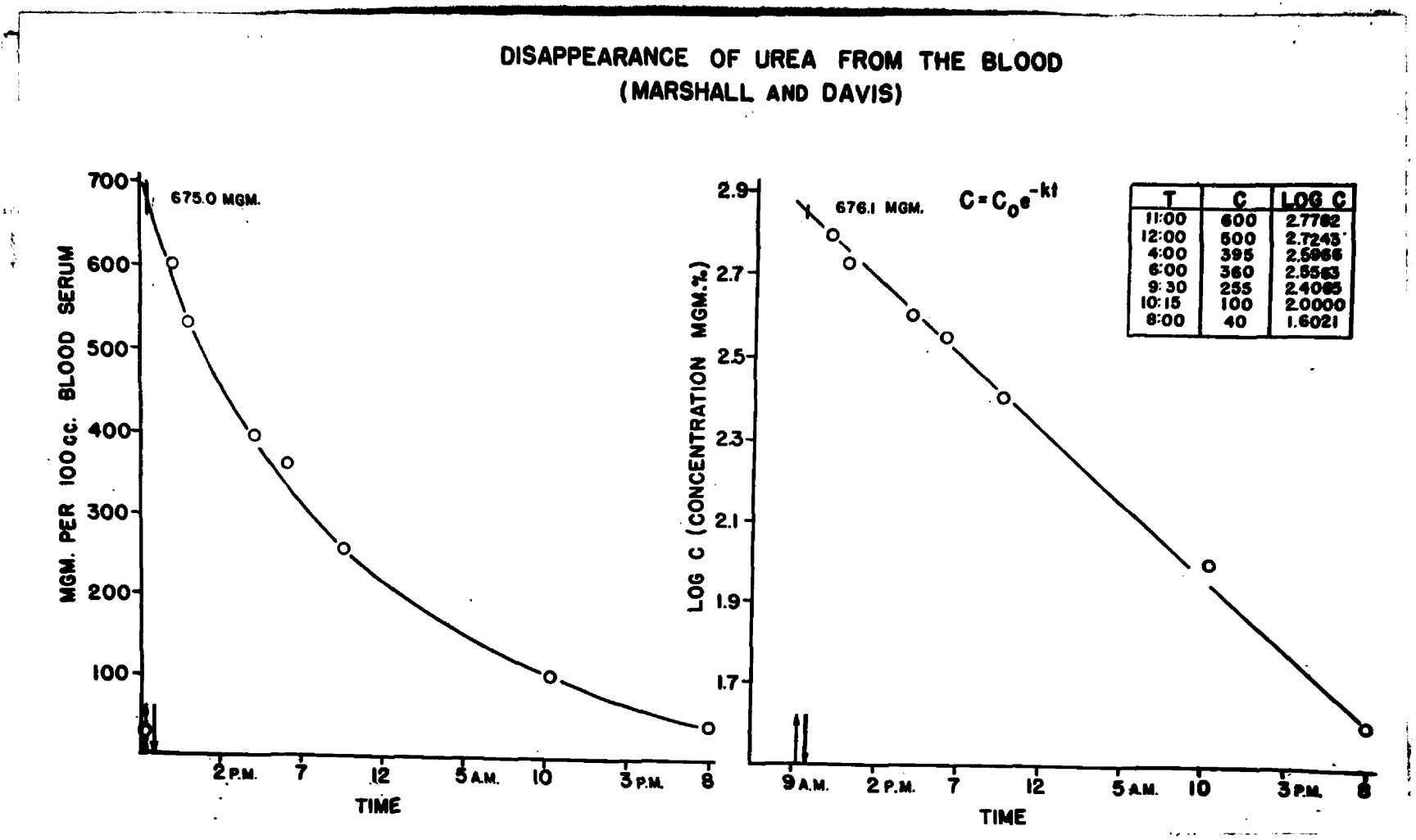


Fig. 6. Two curves showing exogenous urea levels in the blood of a dog over a period of 23 hours. The curve on the left was plotted arithmetically; the one on the right, on a time - log concentration scale. When each curve is extrapolated to the time of injection, comparable results are obtained.

Water available for dilution of sulfanilamide in normal animals

In these experiments the same series of animals were used. A total dose of approximately 800 mgm. of sulfanilamide* was given intravenously. Analysis of a typical curve of the disappearance of sulfanilamide from blood shows that this substance, also, diffuses rapidly during the first hour.** After this period there is a constant rate of disappearance (see Fig. 7). When 55 to 65 mgm. per Kg. have been injected, experiments carried on for periods longer than four hours show the disappearance rate to remain the same. This indicates that intravenously injected sulfanilamide must be uniformily distributed in one to two hours. Results of these experiments show the rate of disappearance to be slow (5 to 9 per cent per hour) in the normal (see Table XIII) and dehydrated dog. In a hydrated animal, however, the rate is somewhat higher (10 to 15 per cent per hour). This slow disappearance rate is advantageous in measuring changes in total body water that are effected deliberately during the course of an experiment (as in demineralization, anesthesia, prolonged hormone injections, etc.).

*Eight-tenths per cent sulfanilamide in 0.5 per cent sodium chloride solution.

**In the time-concentration curves of the sulfanilamide experiments, time is expressed in hours, and concentration, in extinction coefficient (a factor, 5.7, converts the extinction coefficient figure into mgm. per cent).

DISAPPEARANCE OF SULFANILAMIDE FROM THE BLOOD
(3-4 hour experiment)

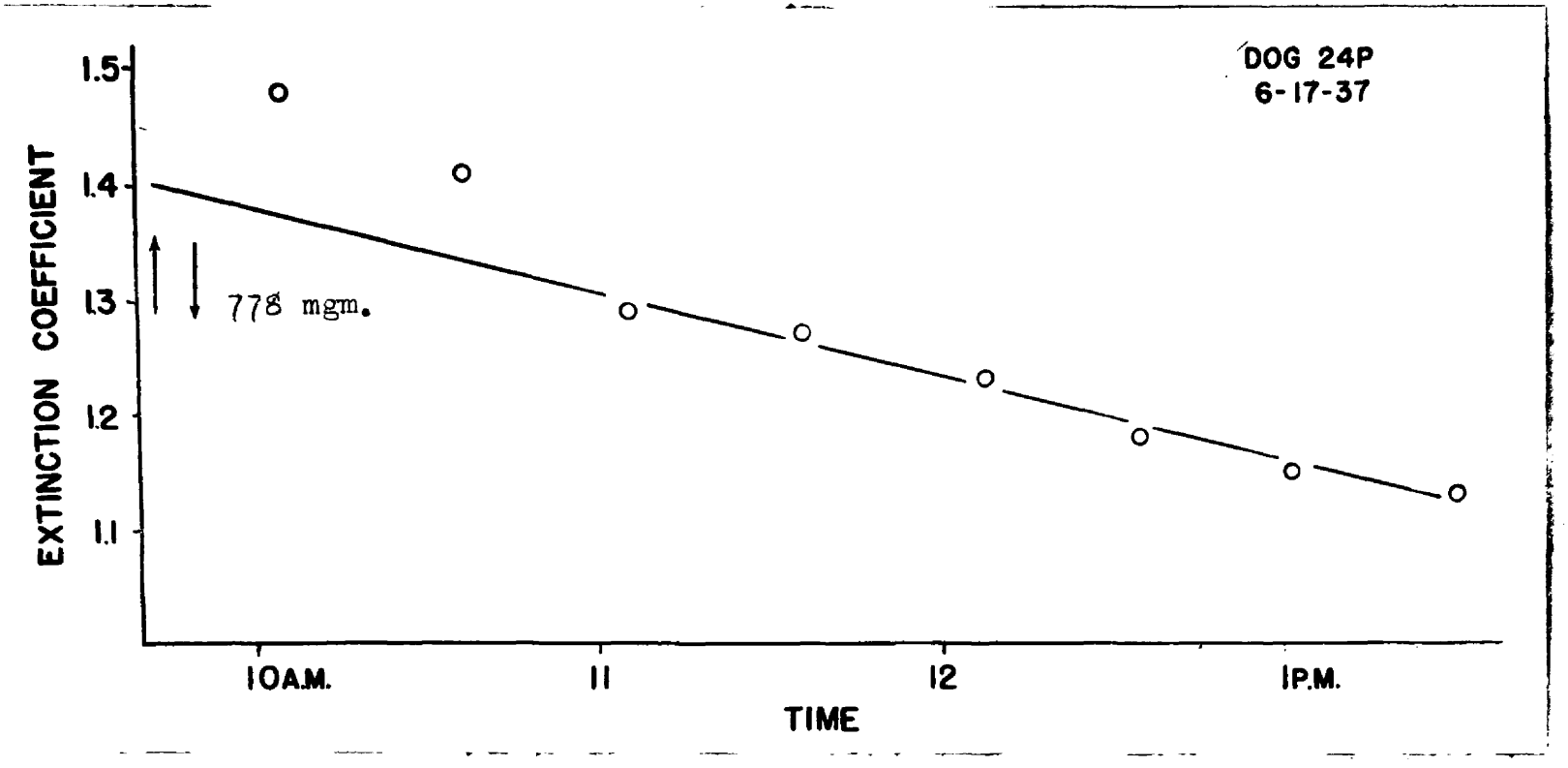


Fig. 7. Curve showing complete distribution one hour after injection. Extrapolation of that portion of the curve attributed to excretion shows a "theoretical" blood level of 7.75 mgm. per cent.

TABLE XIII

WATER AVAILABLE FOR DILUTION OF SULFANILAMIDE IN NORMAL ANIMALS

Date	Dog	Weight in Kg.	Amount Substance Injected Total Mgm.	Mgm./Kg.	Water in Whole Blood Gm./100 Gm.	Disappearance Rate Per Cent per Hour	Total Water-liters Calculated-Blood	Water per cent body weight	Water % of former body weight	Total Water-liters Calculated-urine	Water % body weight	% Recovery Calculated from Urine and Blood Concentration 2-4 hr. period
6/11/37	23P	12.35	796.62	64.5	81.0	9.1	8.37	67.7		9.82	79.5	65.05
6/22/37	23P	12.41	805.7	64.9	82.7	8.28	8.55	68.7		9.06	72.8	65.00
6/17/37	24P	11.90	778.0	65.4	79.2	4.51	7.96	66.8		8.75	73.5	57.70
6/29/37	24P	11.77	805.7	68.5	81.5	6.75	8.21	69.6		8.54	72.5	79.70
2/4/38	24P	14.26	815.0	57.1	79.6	5.50	7.51	52.6	63.6	7.49	52.5	100.00
7/1/37	22P	14.08	757.0	53.8	80.0	7.60	9.47	67.4		9.78	69.5	79.2
3/2/38	22P	16.31	855.0	52.4	77.39	6.77	9.26	56.7	65.7			
7/7/37	29P	14.47	757.0	52.4	79.8	6.31	9.09	62.8		9.17	63.3	95.6
7/22/37	29P	14.21	787.0	55.3	78.3	6.06	9.34	65.6		9.58	67.4	89.75
2/23/38	29P	16.24	855.0	52.6	78.0	5.00	9.02	55.5	63.0	9.40	57.8	73.0

The results (calculated with formula 1) of 10 sulfanilamide experiments have been tabulated in Table XIII. In 7 experiments the available water ranged from 63 to 69 per cent of the body weight, the average being 67.0. Three recent experiments on dogs, 22P, 24P and 29P have been included in the table to show that total water for these animals remained approximately the same over a period of 8 to 9 months, although their weights changed. This in turn is reflected in the water-body weight relation. The change in weight occurred over a period of 2-3 months after the animals were transferred from the University of Maryland Laboratories to those of Columbia University. From the appearance of these adult animals it could be observed that most of the increase in weight had occurred in the form of fat deposits, which were quite apparent in the belly and neck regions.

Table XIII also includes the results calculated from data obtained from the sulfanilamide content of blood and urine (formula 2). To insure comparable results with those obtained from blood disappearance curves, a blood sample was taken simultaneously with the washing of the bladder. In this "excretion method", calculations were based on results of analyses of samples obtained 2-4 hours after the administration of the compound, when distribution was presumably complete. These results are slightly higher (2-3 per cent) and are scattered over a wider range than those calculated from the blood disappearance curves. These higher values may be explained by the following: (1) during the continual excretion

by the kidneys, there is a lag in the redistribution of the sulfanilamide between the tissues and the blood; and (2) since the clearance studies of Marshall et al. (1937b) indicate that tubular reabsorption occurs, it is possible that the renal tubules delay the return of the substance to the blood stream.

When experiments are conducted for periods of 10-12 hours (such as the one recorded on Protocol for Dog 29P and expressed graphically in Fig. 9), the fact that the substance disappears at a percentage rate becomes apparent. However, when the concentration values are plotted exponentially,

$$C = C_o e^{-kt}$$

where C is the concentration at time t, C_o the original concentration, and k the disappearance rate per unit of time, they fall on a straight line (see Fig. 10). This justifies extrapolation to the point of injection and also shows from a comparison of data that linear extrapolation gives approximately the same result. The difference, 0.5 - 1.5 per cent, is within the limits of experimental error. Hence the data of all experiments have been plotted on a linear instead of an exponential scale, except when the values show marked deviation. The results of this and similar experiments show that a 3-4 hour disappearance curve is sufficient for compiling such data.

In Fig. 11 the urine excretion is plotted against blood concentration. It may be seen that extrapolation of the line

PROTOCOL FOR SULFANILAMIDE
(Experiment of Dog 29P)

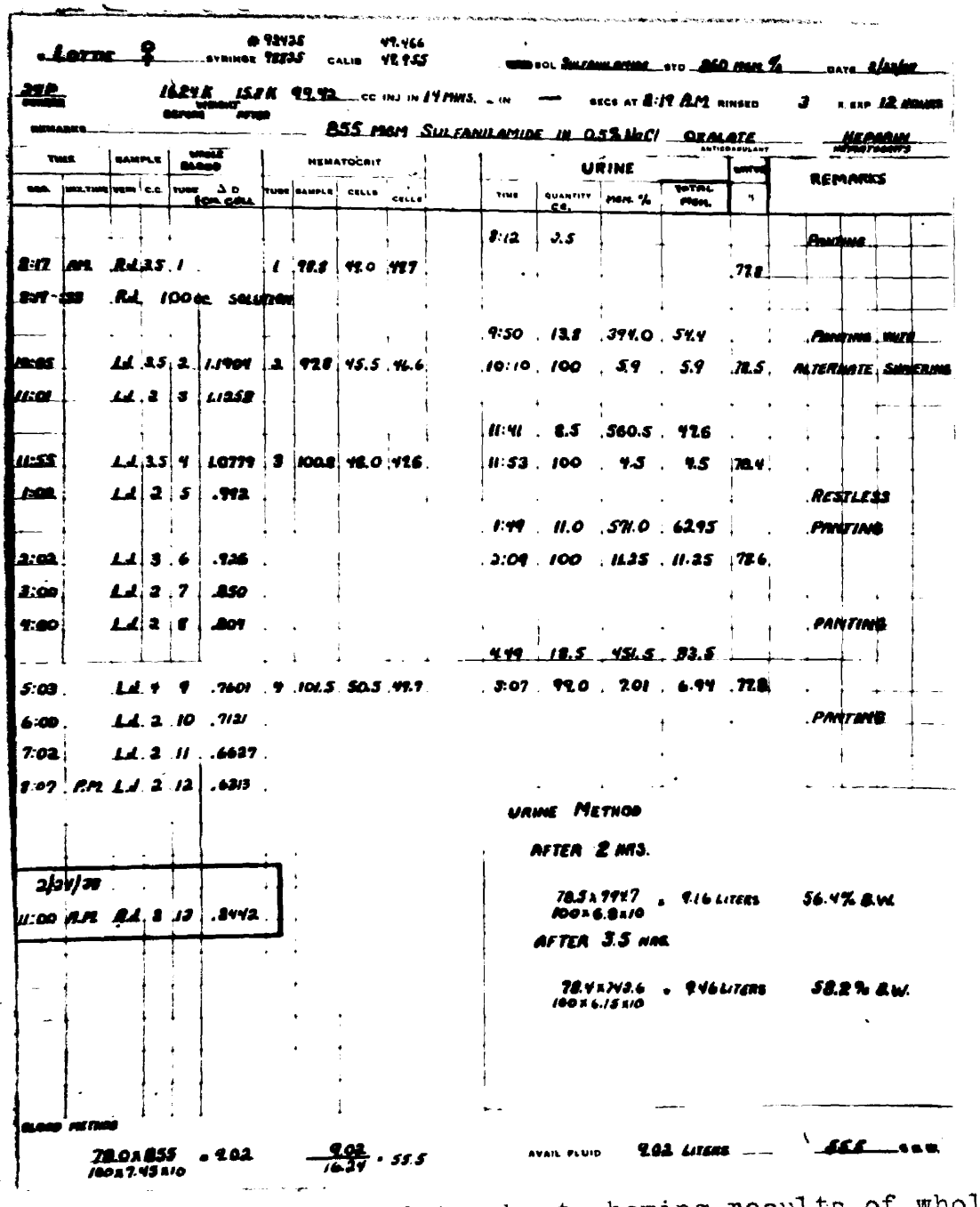


Fig. 8. A typical data sheet showing results of whole blood analysis, expressed as extinction coefficient, hematocrit readings, urine figures in mgm. and water content of blood. Calculations based on formula 1 (blood method) and formula 2 (urine method) show comparable results. The low figures (% B. W.) are attributed to the increase in weight of the animal.

DISAPPEARANCE OF SULFANILAMIDE FROM WHOLE BLOOD
(12 hour experiment)

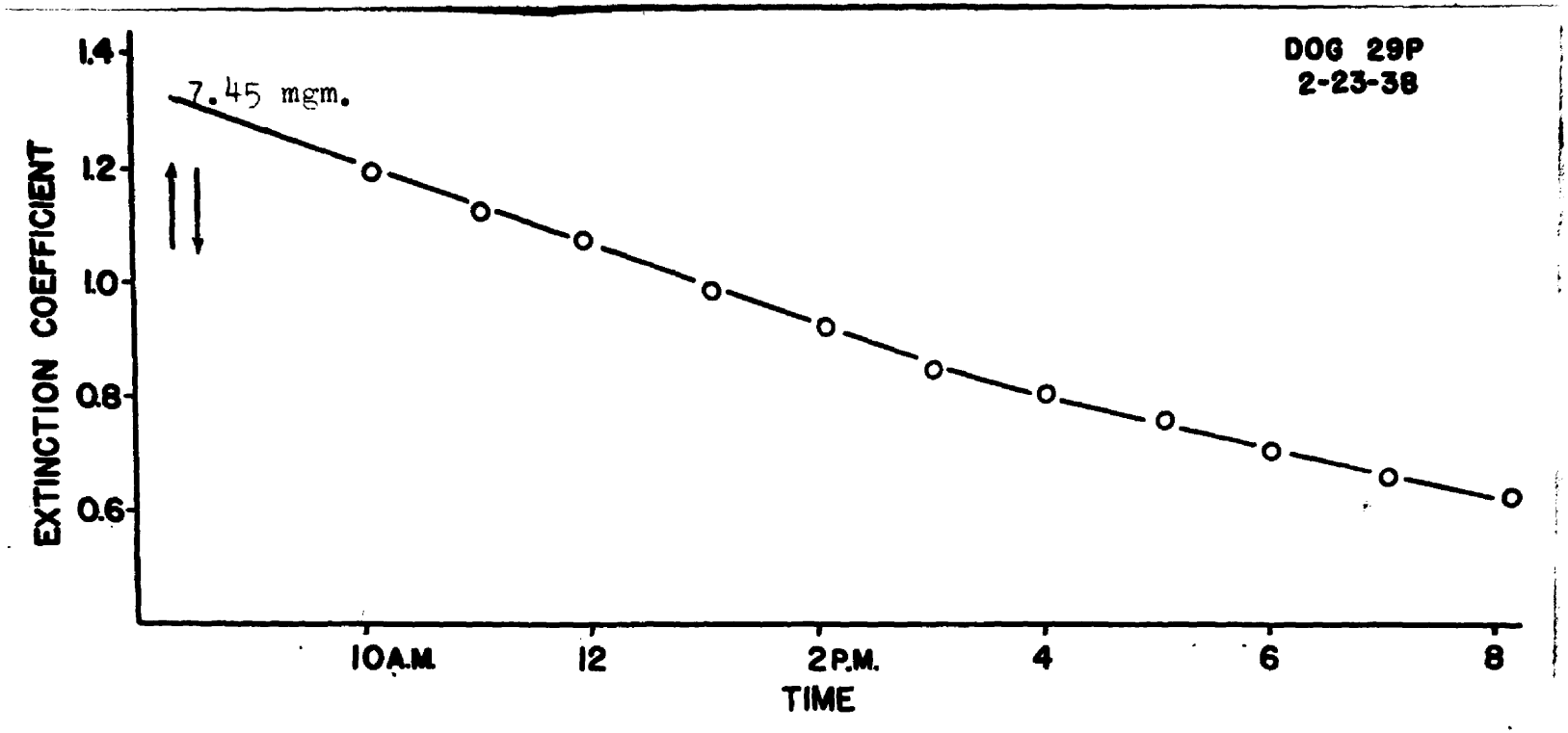
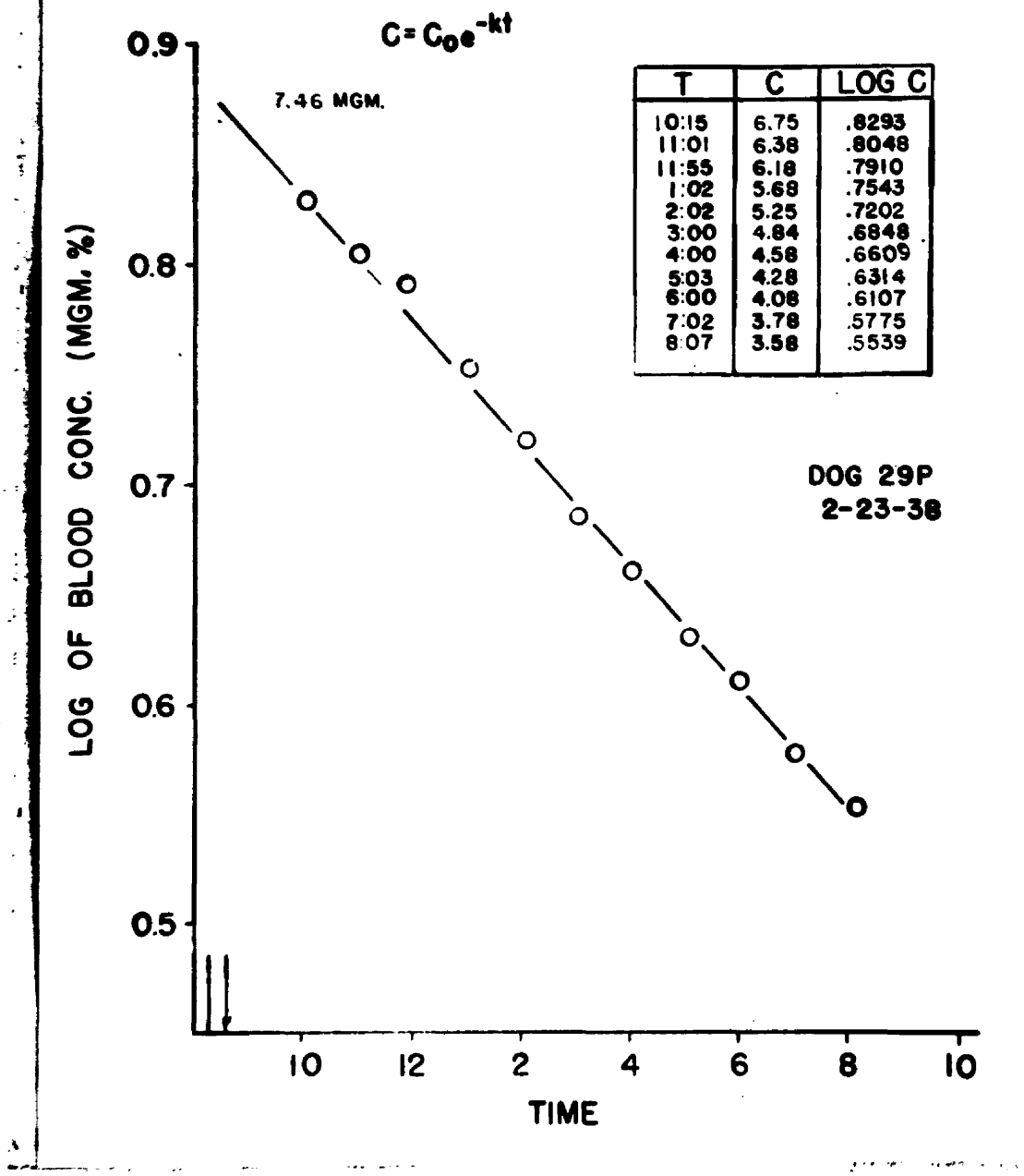


Fig. 9. Curve showing the gradual disappearance of sulfanilamide after an intravenous injection of 855 mgm. The slope of the curve indicates a percentage rate disappearance. Concentration is expressed in terms of extinction coefficient which can be converted into mgm. per cent by the factor, 5.7.

DISAPPEARANCE OF SULFANILAMIDE FROM THE BLOOD
(12 hour experiment)



T	C	LOG C
10:15	6.75	.8293
11:01	6.38	.8048
11:55	6.18	.7910
1:02	5.68	.7543
2:02	5.25	.7202
3:00	4.84	.6848
4:00	4.58	.6609
5:03	4.28	.6314
6:00	4.08	.6107
7:02	3.78	.5775
8:07	3.58	.5539

Fig. 10. Values plotted on an exponential scale, time-log concentration, showing the extrapolated figure in mgm. per cent. This figure agrees closely with that obtained by linear extrapolation in Fig. 9.

SULFANILAMIDE: DISAPPEARANCE FROM THE BLOOD
AND RECOVERY IN THE URINE

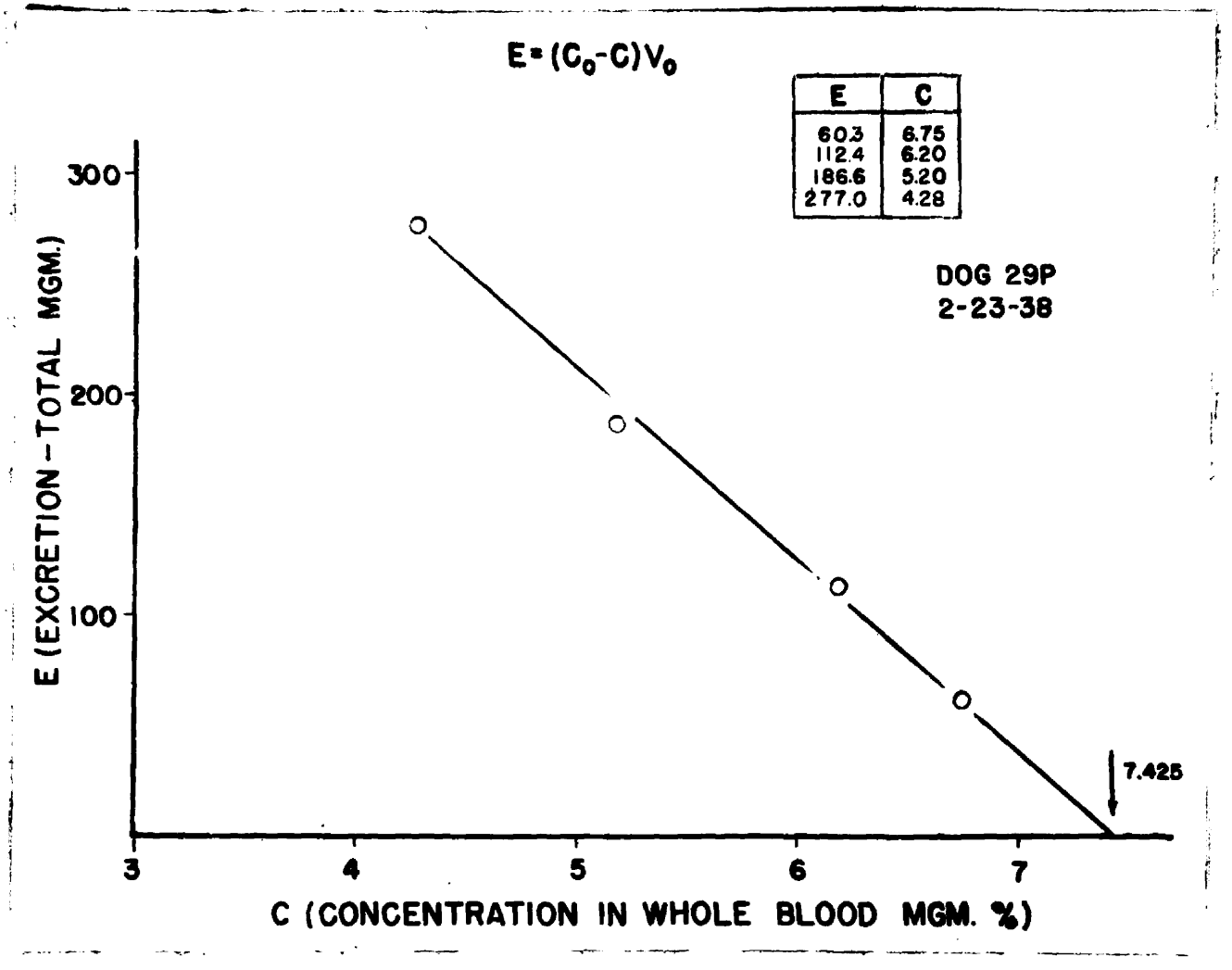


Fig. 11. Curve showing extrapolation to "zero" excretion. This figure obtained (in mgm. per cent) agrees with that from the time-concentration curve. Such comparable results show the blood level to be an index of sulfanilamide content in the body.

to "zero" excretion yields a value for the C_o which agrees exactly with that obtained from extrapolation of the time-concentration curve (Fig. 10). This shows that in the dog there is no conjugation or storage of sulfanilamide.

Simultaneous injections of urea and sulfanilamide in normal animals

The data above give evidence that urea and sulfanilamide are distributed in the same quantity of body water. In order to test this more precisely the two substances have been injected simultaneously in 7 animals and the water available for dilution of each substance has been calculated from formulae 1 and 2.

In these experiments the following factors have been controlled: (1) the water content of whole blood (used for both analyses) was obtained by drying samples to a constant weight at 105°C.; (2) analyses for urea and sulfanilamide content of the solution to be injected were made; (3) animals of approximately the same weight were used; they received no food for 16 hours prior to the experiment, but were given free access to water; (4) two samples of blood were withdrawn 20-30 minutes apart before the solution was injected; these were analyzed for endogenous urea; (5) calibrated syringes were used for injection; (6) 100 cc. of a warm solution containing a known quantity of sulfanilamide and urea, and approximately 500 mgm. sodium chloride were given slowly (in 10-15 minutes) by vein; (7) samples of blood were withdrawn at half hour intervals over a period of 3-5 hours;

(8) the animal was catheterized every hour and the bladder washed three times; the time of urine collections was recorded as the catheter was withdrawn. For the urea analyses a Van Slyke manometric apparatus was used; for the sulfanilamide analyses, Marshall's procedure and a Königs-Marten spectrophotometer.

Fig. 12 shows the disappearance curves of the two substances in a typical experiment. The results of all the experiments are summarized in Table XIV. The available water calculated from formula 1 (disappearance curves) in the seven urea experiments averages 65.3 per cent of the body weight, with a maximum deviation of 3.6; in the six sulfanilamide experiments the average is 66.6, with maximum deviation of 3.8. The calculations based on formula 2 show that for urea the results are somewhat variable (due to changes in the endogenous level) but for sulfanilamide they are consistently 2-3 per cent higher than those obtained by the extrapolation of the time-concentration curve of blood (see Table XIV). The changes in the endogenous urea level are reflected in the last column of the table which shows that in some experiments more urea was excreted than could be accounted for by the disappearance from the blood, and presumably from the body. This figure, percentage recovery, was calculated from a ratio of the total excretion to the total disappearance from the body. The latter was obtained from the decrease in blood level from time of injection to time of excretion, multiplied by total water.

DISAPPEARANCE OF SIMULTANEOUSLY INJECTED UREA
AND SULFANILAMIDE FROM THE BLOOD

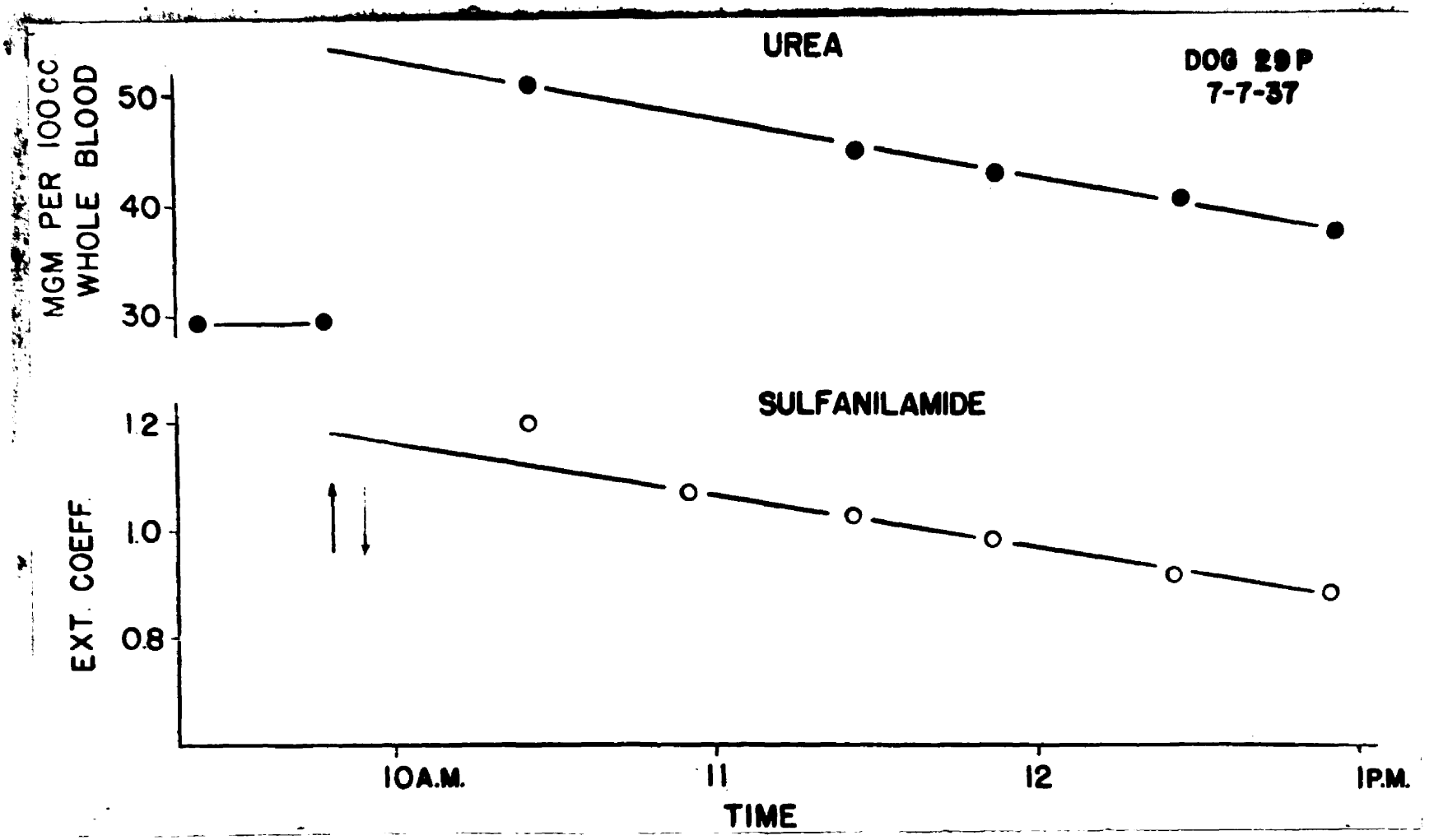


Fig. 12. Curves plotted from the analyses of the two substances showing complete distribution in one hour and the extrapolation of the curves back to the time of injection.

TABLE XIV

AVAILABLE WATER CALCULATED FROM EXOGENOUS UREA AND SULFANILAMIDE IN WHOLE BLOOD (SIMULTANEOUS INJECTIONS)

Date 1937	Dog	Weight in Kg.	Comp.	Injection Mgm./Kg.	Blood Water Gm./100 Gm.	Disappearance %/Hr.	Water calc. from blood liters	% B. W.	Water calc. from urine liters	% B. W.	% Recovery calc. from blood and urine
6/29	24P	11.77	U	248.0	81.5	12.1	7.98	67.8	8.84	75.1	47.0
			S	68.5		8.75	8.21	69.6	8.54	72.5	79.7
7/12	24P	11.82	U	248.0	83.2	8.6	7.22	62.0	7.1	60.6	116.75
7/7	29P	14.47	U	201.0	79.8	10.0	9.59	66.3			
			S	52.4		6.31	9.09	62.8	9.17	63.3	95.6
7/22	29P	14.21	U	207.0	78.3	6.8	9.52	66.85	9.53	66.9	99.5
			S	55.3		6.06	9.34	65.6	9.58	67.4	89.75
6/22	23P	12.41	U	239.0	82.7	15.1	7.67	61.7	7.54	60.7	102.1
			S	64.9		8.28	8.55	68.7	9.06	72.8	65.0
7/1	22P	14.08	U	207.0	80.0	5.3	9.38	66.6	10.19	72.4	81.44
			S	53.8		7.6	9.47	67.4	9.78	69.5	79.2
7/13	7G	13.0	U	225.0	81.0	8.0	8.54	65.6	8.35	64.1	113.3
7/27	55C	9.75	S	77.9	82.0	9.27	6.42	65.8	6.73	69.0	74.7

The agreement in the results obtained simultaneously with urea and sulfanilamide must be regarded as strong evidence that these two substances are diluted by the same quantity of body water. Furthermore, since the water available for their dilution in the dog is essentially the same as the total body water obtained by desiccation (Falck and Scheffer, Pfeiffer, Engels, Thomas, and others) one may safely conclude that the results in Table XIV in reality represent total body water.

Detection of changes in total body water by separate urea and sulfanilamide determinations

In an attempt to determine whether the "dilution method" will detect changes in total water content, hydration and dehydration experiments were performed on unanesthetized dogs. Hydration was produced by administering 1000 cc. of warm salt solution (0.5 per cent sodium chloride) by stomach tube. The results of 6 such experiments using urea as the "water detector" have been tabulated in Table XV. A typical time-concentration curve appears in Fig. 13. Since the administration of large quantities of water produces a water diuresis in 30 to 45 minutes, one might expect a change in the disappearance rate of urea from the blood*.

*In some experiments, this did not occur. Since reinjections of urea were made in order to extrapolate the new time-concentration curve (after hydration), this addition of urea may account for the variation in results obtained.

TABLE XV

WATER AVAILABLE FOR DILUTION OF UREA IN HYDRATION OF TISSUES

Dog	Weight in Kg.	Original Injection Total Mgm.	Water of Serum-Gm. / 100 Gm.	Total Water liters	Water Ingested (0.5% NaCl)	Reinjection Total Mgm.	Water of Serum-Gm. / 100 Gm.	Total Water liters	Difference in liters
24P	11.1	1013.4	94.4	3.12	1.0	1013.4	95.0	4.14	+ 1.02
24P	11.47	1013.4	94.3	6.09	1.0	1013.4	94.6	7.00	+ 0.91
24P	11.5	3957.6	94.4	6.91	1.0	none	95.3	8.57	+ 1.66
29P	14.5	1013.4	94.0	5.48	1.0*	1013.4	95.1	5.14	- 0.34
29P	14.2	1013.4	95.1	8.05	1.0	1013.4	95.9	9.57	+ 1.52
25P	11.36	1013.4	94.5	5.20	1.0	1013.4	95.0	6.60	+ 1.40

*Subcutaneous injection of 1.5 cc. pitressin before administration of water.

DISAPPEARANCE OF UREA FROM THE BLOOD
(Hydration experiment)

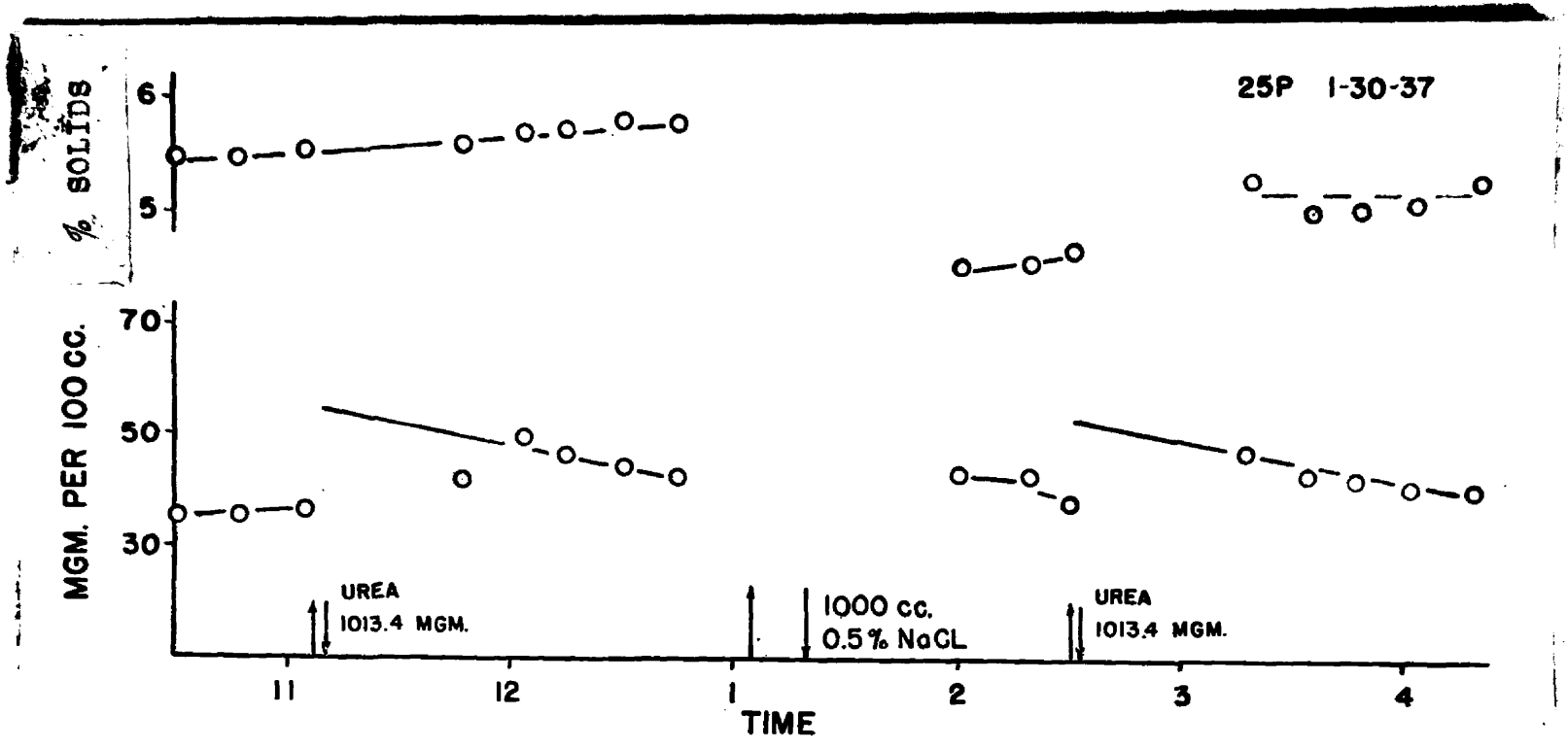


Fig. 13 Curves of urea and solid content of blood showing the changes that occur after the administration of a saline solution and a re-injection of urea, permits extrapolation of the new curve and the calculation from formula 1.

If blood samples are analyzed and an accurate record of urine volume and its urea content can be obtained before and after the hydration, the results should indicate the change in the water content of the body. Such a method would certainly not be quantitatively accurate, since changes in the endogenous concentration can not be determined (suggested by Marshall and Davis, 1914). This illustrates the desirability of comparing urea with a substance foreign to the body in order to determine the significance of changes in the endogenous level of urea.

In two hydration experiments a known amount of sulfanilamide was injected and after distribution had proceeded for one hour, several samples of blood and urine were taken during the course of two hours. At the end of this period 1.0 to 1.5 liters of 0.5 per cent sodium chloride solution were given slowly (20-30 minutes) by stomach tube. Samples of blood and urine were taken one-half to one hour following the administration of the fluid. The time-concentration curve of sulfanilamide in the blood after hydration follows a straight line, although the rate of disappearance has increased to approximately 14 per cent per hour (see Fig. 14). The curve is valuable only in the control period for a comparison of results obtained by formulae 1 and 2. In hydration, extrapolation of the new slope of the curve would not only indicate greater dilution, but also a lower circulating level of the compound, since a certain quantity has been excreted before and during the ingestion of water. Re-

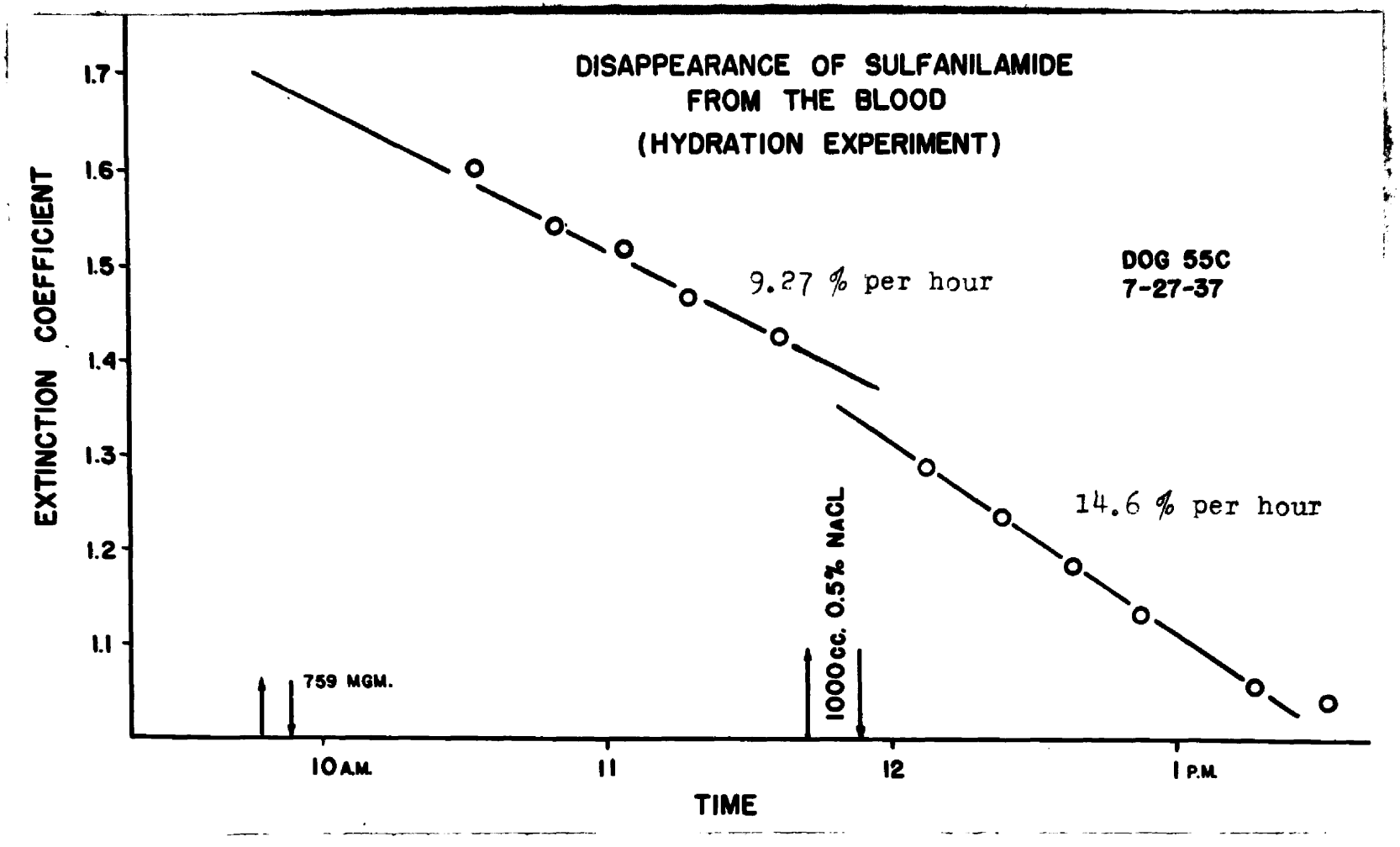


Fig. 14. Curves showing the change in rate of disappearance in blood after ingestion of a hypotonic saline solution. Calculations of the change in total water content have been based on the blood concentrations at 11:28 A. M. and 1:27 P. M., when urine was also removed. (Formula 2 used for calculations).

injections following hydration might permit extrapolation of a new curve.

In the calculations of the present sulfanilamide experiments, in which there were no re-injections, urine excretion figures and single blood samples have been used for dogs 55C and 29P (see Table XVI). It is apparent that water added to the body is available for the dilution of sulfanilamide.

On the mechanisms of dehydration the work of many investigators (Marriott, 1923, Keith, 1923 , 1924a, 1924b, Gamble, 1929, 1930, Underhill and Fisk, 1930, Drake, McKhann, and Gamble, 1930, Wiley and Wiley, 1933, Davis, 1934, Hamilton and Schwartz, 1935, and others) is controversial, especially with regard to: (1) which fluid compartment contributes first; (2) which contributes to the fullest extent toward maintaining a constant blood volume; and (3) the extent of the metabolism of solids and their possible contribution of water to the body in the later stages of dehydration. In the dehydration which can be produced by the intravenous administration of sucrose (Keith, 1923), there was a rise in endogenous urea. Any attempt to correct for this by re-injection of urea is not a solution to the problem, since the kidneys after this rapid dehydration process are no longer eliminating urine.* Excretion by the kidneys is resumed when the extracellular water content is increased by administration of salt and glucose solutions.** Therefore, some other method of dehydration

*One such experiment after the intravenous re-injection showed a gradual rise in the time-concentration curve of urea instead of a fall.

**These solutions were given intravenously and orally to revive the animal.

TABLE XVI

DETERMINATION OF WATER CONTENT BY SULFANILAMIDE METHOD

Hydration Experiments*

Dog	Weight in Kg.	Dose Mgm. / Kg.	Fluid Intake in l.	% Water in Blood	Total Water Liters	Water-% B. W.
55C						
Control	9.75	77.9		82.0	6.73	69.0
Hydrated			1.0	83.5	8.00 + 1.27	81.2
29P						
Control	14.21	55.3		78.3	9.62	67.6
Hydrated			1.50	78.7	11.21 + 1.59	79.0

Dehydration Experiments**

24P						
Control	11.77	68.5		83.2	8.21	69.6
Dehydrated (6 days)	10.39 - 1.38	73.4		79.0	6.77 - 1.44	65.2
7G						
Control	13.0	61.2		81.0	8.46(?)	65.0(?)
Dehydrated (7 days)	11.49 - 1.51	66.4		78.5	7.08 - 1.38	61.65

*Water has been calculated in the hydration experiments by the urine method, since the control and hydrated procedures were carried out in the same experiment with a single dose of sulfanilamide. (using formula 2)

**Water has been calculated in the dehydration experiments by the blood disappearance method, since the control and the dehydration were carried out as two separate experiments. (using formula 1)

had to be considered. The removal of food and water for five days results in complications, since not only a decrease in water content occurs, but also a parallel loss of solids (Drake, McKhann and Gamble, 1930). Table XVII shows the effect of water deprivation on Dogs 24P and 7G determined with urea. It may be seen that the endogenous urea level has increased out of all proportion to any of the other recorded changes. To what extent it may further increase during the course of the experiment can not be determined. This change is the explanation in part for the low values obtained during dehydration. Such a conclusion can be confirmed by a comparison of these and the sulfanilamide experiments. Table XVI shows the results of similar experiments using sulfanilamide. The decrease in water content is comparable with the loss in total body weight. More data must be collected before conclusions can be drawn about the relative amounts of water and solids lost. Nevertheless, the experiments indicate that sulfanilamide may be useful for the detection of changes in total body water in living animals.

Simultaneous determinations of sulfanilamide, sodium thiocyanate and the blue dye T-1824 in blood

One of the chief aims of this investigation was to devise a method for determining total body water, which might be carried out in conjunction with determinations of plasma volume and of fluid available for the dilution of sodium thiocyanate (approximately equivalent to the total extracellular fluid). Combined determinations of this sort would

TABLE XVII

WATER AVAILABLE FOR DILUTION OF UREA IN DEHYDRATION (6-7 DAYS)

Dog	Weight-Kg. (1)	(2)	Change	Endogenous Urea level (1)	(2)	Dose Mgm./Kg. (1)	(2)
24P	11.51	9.65	- 1.86	27.0	57.6	344	210.0
24P	11.82	10.39	- 1.43	38.4	38.5	248	284.0
7G	13.00	11.49	- 1.41	37.5	29.5	225	256.5

Water of blood-Gm. % (1)	(2)	Total water in liters (1)	(2)	Change	Water % B. W. (1)	(2)
94.4	92.3	6.97	4.53	- 2.44	60.6	47.0
83.2	79.0	7.27	6.55	- 0.72	62.0	63.0
81.0	78.5	8.54	6.18	- 2.36	65.6	53.9

(1) Before dehydration
(2) During dehydration

yield the only means at present available for estimating changes in the volume of intracellular fluid in the living animal. Furthermore, these methods would make it possible to investigate disturbances in the internal fluid balance in a variety of experimental and pathological conditions.

Gregersen and Stewart (1938) have already shown that determinations of plasma volume with the dye T-1824 can be combined with the thiocyanate dilution method. It remains to be seen whether or not these two methods can be combined with the sulfanilamide determinations. To test this point a series of experiments were carried out on blood in vitro.

Dye and sulfanilamide were added to whole blood and thiocyanate and sulfanilamide to another sample. Results in Table XVIII show that the thiocyanate interferes with complete recovery of the sulfanilamide. Table XIX is presented to show that thiocyanate is not affected by the sulfanilamide and that it is not altered in its chemical reactions. Thiocyanate on the other hand interferes either with the diazotization or the coupling of sulfanilamide to form the red azo dye. Results tabulated in Table XX indicate that thiocyanate interferes with the coupling process. The effect is proportional to the concentration of thiocyanate present in the blood sample.

Attempts were made to remove the thiocyanate before analyzing for sulfanilamide. Table XXI shows the results obtained when thiocyanate is precipitated with a silver salt and the filtrate analyzed for sulfanilamide. Better results

TABLE XVIII

SULFANILAMIDE ANALYSIS

10 cc. Sample-Whole blood containing			Results	
Sulfanilamide 20 mgm. %	Dye 1 %	Thiocyanate 5 %	Theoretical Mgm. %	Actual Mgm. %
6.0 cc.	0.05	0	6.25	6.3
6.0	0	0.15	6.25	3.85
6.0	0	0	6.25	6.12

TABLE XIX

SULFANILAMIDE VERSUS THIOCYANATE

Study of Effect of Sulfanilamide on Thiocyanate Analysis

Sulfanilamide	Thiocyanate	Results Theoretical Thiocyanate	Actual Thiocyanate
15 mgm.%	40 mgm.%	5.0	5.08
15	32	4.0	4.04
15	24	3.0	3.09
15	16	2.0	2.025

Study of Effect of Thiocyanate on Sulfanilamide Analysis

		Sulfanilamide	
10	3	5.0	3.67
15	3	7.5	4.85
20	3	10.0	6.50

TABLE XX

STUDY OF INCREASE IN QUANTITY OF COUPLING AGENTS

Study of Increase in Nitrite on the effect of Thiocyanate on Sulfanilamide Analysis

Sulfanilamide	Thiocyanate	Nitrite	Results Theoretical	Results Actual
			Sulfanilamide	
15	0	.1	7.5	8.2
15	3	.2	7.5	4.2
15	3	.3	7.5	4.25

Study of Increase in Dimethyl-α-naphthylamine on the effect of Thiocyanate on Sulfanilamide Analysis

Sulfanilamide	Thiocyanate	Dimethyl-α-naphthylamine		
15	3	0.5	5.62	3.7
15	3	1.0	5.62	5.75

TABLE XXI

THE EFFECT ON SULFANILAMIDE ANALYSIS OF REMOVAL OF THIOCYANATE WITH SILVER SALT; REMOVAL OF EXCESS SILVER WITH ACID

Sulfanilamide	SCN	$AgNO_3$	HCL	Results Theoretical	Results Actual
100	3	1	0	44.4	51.75
100	3	1	1	29.6	28.9

were obtained, however, when the excess silver was removed by the addition of hydrochloric acid. This latter procedure yields complete recovery of the sulfanilamide in the form of the azo dye, but experimental error is increased and the procedure is a tedious one.

Recent spectrographic studies* (Gregersen and Painter, 1938) of recrystallized sulfanilamide in water have shown an absorption band in the ultraviolet spectrum from 230 to 290 mμ with maximal absorption at 259.5 mμ.** The spectral absorption curves of two solutions (0.5 mgm. per cent and 1.0 mgm. per cent) appear in Fig. 15. Since water and ethyl alcohol show no absorption in this region of the spectrum, whole blood samples containing sulfanilamide can be extracted with alcohol, as described in Marshall's original method (Marshall et al., 1937a) for the removal of proteins in sulfanilamide analysis. This extract was evaporated and the residue dissolved in a known quantity of water. After filtration, this solution was relatively free of fats and showed maximal absorption at 259.5 mμ. The quantity of sulfanilamide present can be determined by the extinction coefficient at the peak of absorption. In a series of

*Hilger quartz spectrograph, equipped with a Spekker photometer and a hydrogen discharge tube for light source, was available for this work through the kindness of Dr. Hans Clarke.

**It was interesting to find reports of sulfanilamide as a photosensitizing agent (Newman, 1937) and as source of dermatitis on exposed skin areas (Menville and Archimard, 1937, Goodman and Levy, 1937, and Frank, 1937). These effects are probably related to the absorbing capacity of the drug in the ultra-violet.

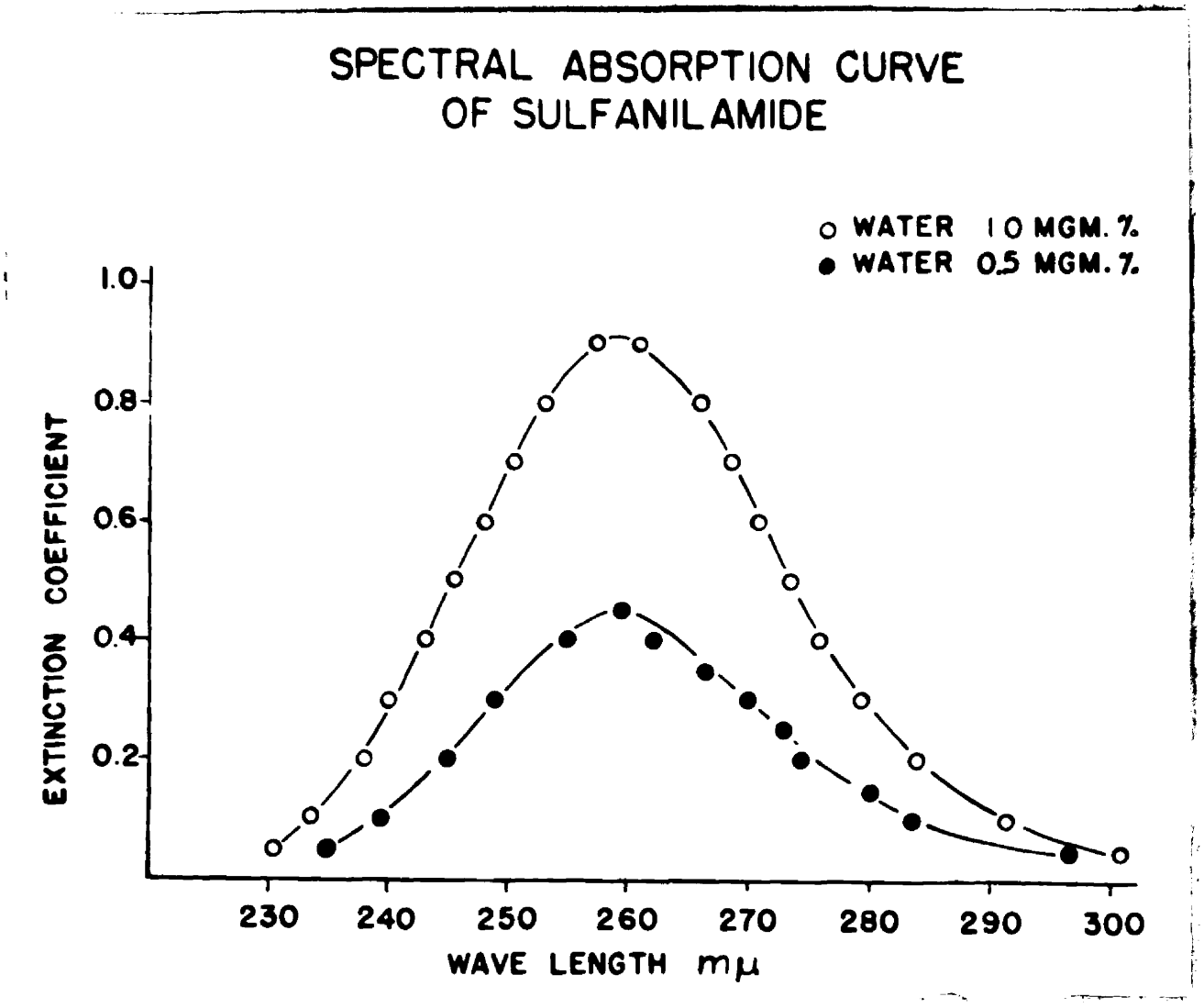


Fig. 15. Curves showing the peak of absorption at 259.5 mμ for both solutions. These aqueous solutions contain recrystallized sulfanilamide.

analyses the extinction coefficient was found to be directly proportional to the concentration of the substance in aqueous solution. Fig. 16 shows this to be true also for sulfanilamide added to whole blood. The spectrographic method should be suitable for the analysis of sulfanilamide in blood when dye and thiocyanate are present in the sample. This was demonstrated by the following: two samples of blood, one containing dye, thiocyanate and sulfanilamide, and the other, the same quantity of sulfanilamide were extracted with alcohol and analyzed spectrographically. The extinction coefficients were identical within the limits of experimental error.

Conclusion

Intravenously injected urea and sulfanilamide reach equilibrium between blood and tissues of the dog in approximately one hour.

The water available for the dilution of these substances varies from 62.0 to 69.0 per cent of the body weight, the average being 65.3 for urea and 66.6 for sulfanilamide. Since these figures agree closely with those obtained by the desiccation method for the same animal, it is concluded that they represent total body water.

Fluctuations in the endogenous urea level interfere with accurate measurements of changes in the total body water with this substance. Sulfanilamide, a substance foreign to the body, is satisfactory for measuring changes in total body water.

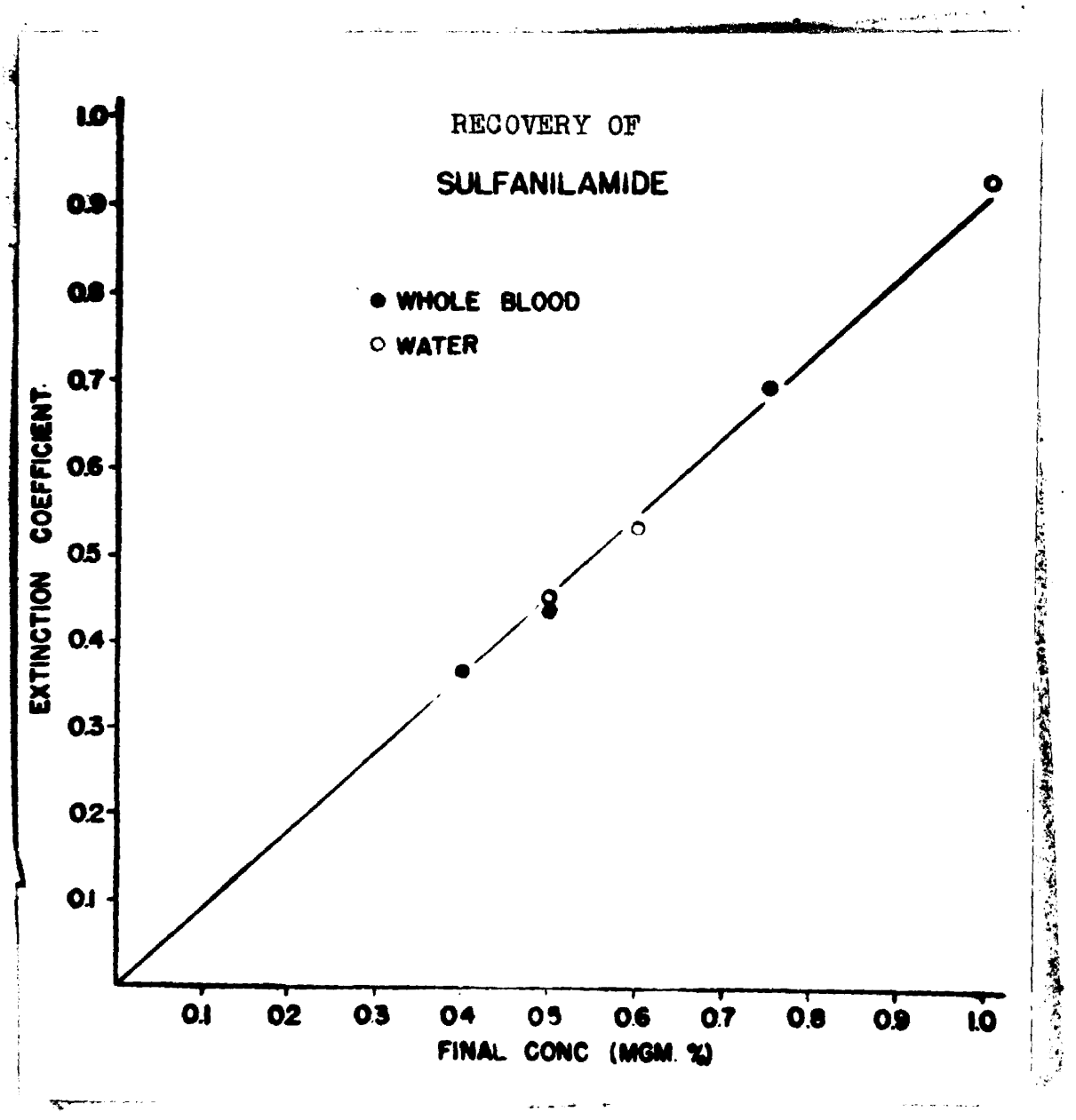


Fig. 16. Curve showing complete recovery of free sulfanilamide from whole blood by spectrographic analysis.

Thiocyanate interferes with the chemical analysis of sulfanilamide. When thiocyanate is present, as for example in determinations of "available fluid" and total body water, sulfanilamide can be determined spectrographically in the alcohol extracts of blood.

SUMMARY

A survey of the literature shows that the total water content of man and animals has been determined chiefly by the desiccation method. Approximately 65 per cent of the body weight is attributed to water. Since 1920, however, osmotic pressure changes, resulting from the addition to the body of non-diffusible ions, have been the means for indirectly determining the water content of the body. The present investigation is concerned with diffusible substances that might be distributed throughout all the body water. Then, the water available for the dilution of such a substance should indicate the total body water. After a study of the chemical nature, the toxicity and pharmacological action, and the quantitative analytical methods of twelve substances, urea and sulfanilamide appeared to be the most satisfactory.

In separate experiments with urea and sulfanilamide given intravenously, the time-concentration curves for blood indicate that equilibrium between blood and tissues is approached about an hour after injection.* Beyond this point the disappearance rate is constant for several hours (4-5). For exogenous urea (200-350 mgm. per Kg.) the disappearance rates ranged from 9 to 15 per cent per hour, for sulfanilamide (55-65 mgm. per Kg.) from 6.0 to 9.1 per cent.

*Blood and urine were analyzed for urea by the Van Slyke gasometric method; for sulfanilamide by a spectrophotometric modification of Marshall's chemical method.

On the assumption that linear extrapolation back to the time of injection gives the concentration which would be obtained if distribution were complete and none of the substance had been excreted, the "available water" calculated from twelve urea experiments averaged 62.9 per cent of the body weight and from seven experiments with sulfanilamide 66.9 per cent. Both substances are therefore distributed in approximately the same volume.

This conclusion is confirmed by injecting measured amounts of urea and sulfanilamide simultaneously. The dilution (corrected for disappearance as above) is, within the limits of experimental error, the same for both. In six experiments the "available water" calculated from urea (using formula 1) ranged from 62 to 68 per cent of the body weight, the average 65.3; for sulfanilamide the range was 63 to 69, the average 66.0. It is probable that these values represent total body water since the relative amount of sulfanilamide or urea taken up by the tissues per unit weight is with minor exceptions directly proportional to their water content (Marshall). Furthermore, the figures agree closely with the total body water determined by desiccation (literature).

If the calculation (using formula 2) of "available water" is based on a single blood sample taken during the second hour (when distribution is presumably complete) and on the amount of sulfanilamide or exogenous urea remaining in the body (total injected minus that recovered in urine), the values vary for urea, but for sulfanilamide they are consistently 2-3 per cent higher than those obtained from the disappearance

curves.

The presence of thiocyanate interferes with the chemical analysis of sulfanilamide in the blood. When thiocyanate is present, as for example in determinations of "available fluid" and total body water, sulfanilamide can be determined spectrographically in the alcohol extracts of blood.

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