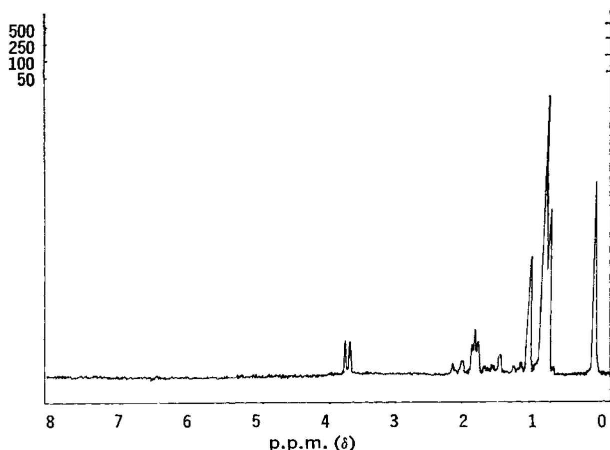

Figure 7—*NMR spectrum of Peak 6A of essential oil of* Anemopsis californica *from polyethylene glycol.*

The coupling constant ($J = 9.1$) also suggested an isolated aromatic proton. Thus the substitution pattern was seen to be 1:2:4. Among the possible structures, thymolmethylether was the immediate choice on biogenetic basis; thymol had been shown to be present in the oil in appreciable quantities. The IR and NMR spectra of synthetic thymolmethylether were identical with those of Peak 9.

Other compounds identified from the essential oil are given in Table II. Among these, β-pinene was identified by the comparison of peak IR with a published spectrum (8). The total fraction under Peaks 20 to 25 was collected in CCl_4. The IR spectrum of this sample showed that these six compounds are hydrocarbons.

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Biopharmaceutical Studies of Aminoethanesulfonylphenetidine I

SHUN-ICHI NAITO and KAZUO FUKUI

Abstract □ Blood levels of aminoethanesulfonylphenetidine (taurinophenetidine) in rabbits were determined and binding of the drug with rabbit serum protein *in vivo* and *in vitro* was examined. Metabolites of taurinophenetidine in rabbit urine were separated into four compounds, *p*-aminophenol, *p*-acetamidophenol, *p*-phenetidine, and unchanged taurinophenetidine. Metabolites of acetophenetidine were also investigated. Glucuronide in urine of rabbit ingesting taurinophenetidine is the conjugated form of *p*-aminophenol.

Keyphrases □ Aminoethanesulfonylphenetidine—biochemistry □ Metabolites, aminoethanesulfonylphenetidine—rabbit plasma, urine □ TLC—separation, identification □ Colorimetric analysis—spectrophotometer

Aminoethanesulfonic acid which is expected to separate from aminoethanesulfonylphenetidine when ingested, is one of the essential amino acids, in human subjects and has also surface-active property. It is widely known that *p*-phenetidine has antipyretic and analgesic actions, nevertheless, its toxicity must be severe. Aminoethanesulfonylphenetidine, the condensation product of aminoethanesulfonic acid and *p*-phenetidine, was prepared in order to obtain a more potent chemical than acetophenetidine with less side effects. Detailed actions of this new chemical cannot be predicted from the chemical structure without extensive research, since it is often observed that small changes in a chemical structure change pharmacological and biochemical behaviors of the chemical essentially, additionally, and extensively.

In the previous paper (1), stabilities of aminoethanesulfonylphenetidine (hereafter abbreviated as taurinophenetidine) stored at different temperature and pH were reported. It might be said that taurinophenetidine is stable in either solution or powder form to temperature, pH or moisture.

The purpose of this paper is to discuss some biopharmaceutical aspects of taurinophenetidine.

EXPERIMENTAL

Metabolites of Taurinophenetidine in Blood—A mixture of 0.5 ml. of rabbit plasma, taken 0.5 hr. (peak time of blood level) after

oral ingestion of 350 mg./kg. of taurinophenetidine, and 0.5 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. The mixture was neutralized with 0.5 ml. of 5 *N* Na_2CO_3 solution and 1.5 ml. of H_2O, and 7 ml. of $CHCl_3$ were added. This mixture was shaken vigorously for 1 hr., and centrifuged. After evaporation of the $CHCl_3$ layer, the residue was mixed with 50 μl. of EtOH, and 10 μl. of this mixture was spotted for TLC. Blood before ingestion of the drug was treated by the same procedure as a control. The experiment was made with two rabbits.

TLC: solvent, benzene: acetone (2:1); adsorbent, diatomite, (Kieselgel G); color developer, 5% silver nitrate solution; R_f values, in treated plasma 0.17, 0.23, 0.40, 0.75; in control plasma 0.17, 0.40, 0.75; R_f value of taurinophenetidine, 0.23.

Determination of Taurinophenetidine in Blood—*(Hereafter Abbreviated as Modified Folin Method)*—To 1 ml. of plasma in an ice-cold centrifuge tube, 2 ml. of EtOH was added and the mixture was stirred thoroughly. After standing for 15 min. under cooling with ice-water, 0.5 ml. of the supernatant from centrifugation was mixed with 0.5 ml. of H_2O, 0.5 ml. of 0.1% $CuSO_4$ solution, 3 ml. of 12.5% Na_2CO_3 solution, and 0.5 ml. of phenol reagent (1:1) (2). After 1.5 hr. at room temperature, absorbance of a clear solution obtained by centrifugation was determined at 720 mμ. The mixture of 0.9 ml. of normal rabbit plasma and 0.1 ml. of taurinophenetidine solution of known concentration was treated as above, to prepare a calibration curve.

Binding of Taurinophenetidine with Rabbit Serum Protein In Vitro—Absorbance (*a*) of the solution after an addition of 0.1 ml. of taurinophenetidine solution (250 mg. %) to 0.9 ml. of normal rabbit serum was measured immediately by the modified Folin method.

Absorbance (*b*) of the solution after an addition of 0.1 ml. of H_2O to 0.9 ml. of normal rabbit serum was also determined by the same method. Difference between (*a*) and (*b*) is the amount of taurinophenetidine in serum which was 258 mcg.

Absorbance (*c*) of the mixed solution of 0.1 ml. of taurinophenetidine solution (250 mg. %) and 0.9 ml. of normal rabbit serum kept in an incubator at 37 ± 2° for 24 hr. was determined by the modified Folin method. Absorbance (*d*) of the mixed solution of 0.1 ml. of H_2O and 0.9 ml. of normal rabbit serum kept in an incubator at 37 ± 2° for 24 hr. was determined by the same method. Difference between (*c*) and (*d*) is the amount of free taurinophenetidine in serum kept at 37 ± 2° for 24 hr., which was 84 mcg. Accordingly, the difference (258–84 = 174 mcg.) means the amount of taurinophenetidine bound with protein and the ratio to total taurinophenetidine was about 67%. The absorbance (*e*) and (*f*) were determined after 0.5 ml. of the plasma showing the absorbance (*c*) or (*d*) was mixed with 0.5 ml. of 5 *N* HCl and kept at 37 ± 2° for 1–2 hr. The difference between (*e*) and (*f*) shows the amount of total taurinophenetidine, free and bound with protein. The fact that the difference of (*e*) − (*f*) almost coincided with that of (*a*) − (*b*) shows that treatment with 5 *N* HCl was enough to prohibit the protein binding with taurinophenetidine.

Binding of Taurinophenetidine with Rabbit Serum Protein In Vivo—A mixture of 0.5 ml. of serum from rabbits ingesting the drug and 0.5 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1.5 hr. The mixture was treated by the modified Folin method for the determination of total taurinophenetidine, both free and conjugated with protein. Free taurinophenetidine in 0.5 ml. of serum was determined without treatment with 5 *N* HCl by the modified Folin method. No difference was observed between free and total taurinophenetidine content in serum of rabbits ingesting the drug in 350-mg./kg. doses as shown in Table I.

Separation of Metabolites of Taurinophenetidine in Urine—About 500 ml. of urine from four rabbits ingesting 450 mg./kg. of taurinophenetidine was submitted to freezing evaporation. The yield of the residue (*A*) was about 13.2 g. The residue (*B*) of urine was obtained from four rabbits which did not receive the drug served as a control.

A mixture of 500 mg. of the residue (*A*) or (*B*) and 1 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. After the addition of 1 ml. of 5 *N* Na_2CO_3 solution, the Mixture *A'* from (*A*) or *B'* from (*B*) was submitted to TLC. On the other hand, a mixture of 2 ml. of H_2O and 500 mg. of the residue (*A*) or (*B*) was also submitted to TLC. No difference was observed in the number of spots between the mixture (*A*) and *A'* or between (*B*) and *B'*.

TLC (I): solvent, benzene and $(CH_3)_2CO$ (2:1); adsorbent, diatomite (Kieselgel G); color developer, 5% $AgNO_3$ solution; R_f values of the mixture (*A*) or *A'*; 0.23 (taurinophenetidine), 0.33 (*p*-acetamidophenol), 0.50 (*p*-aminophenol), 0.85 (*p*-phenetidine), R_f values of the mixture (*B*) or *B'*, none.

TLC (II): solvent, butyl alcohol, isopropyl alcohol and 28% NH_4OH (2:1:1); adsorbent, diatomite (Kieselgel G); color developer, 0.5% 2,4-dinitrofluorobenzene in ethyl acetate; R_f values of the mixture (*A*) or *A'*; 0.47 (taurinophenetidine), 0.65 (*p*-aminophenol) and 0.85 (*p*-phenetidine); R_f values of the mixture (*B*) or *B'*, none.

p-Acetamidophenol was not so sensitive to the color reaction with 2,4-dinitrofluorobenzene solution.

Table I—Plasma Level of Taurinophenetidine (mcg./ml.) Following its Oral Administration to Rabbits

Rabbit No.	Body Weight, kg.	Dose, mg./kg.	Hours After Dosing 0.5	1.0	2.0	4.0	7.0
1	2.0	250	42	64	61	32	16
2	1.9	—	40	80	56	44	36
3	2.0	—	55	67	67	44	35
4	2.0	—	58	70	59	62	34
Mean value	2.0	—	49	70	61	46	30
5	2.3	350	74	96	179	72	80
6	2.5	—	80	148	76	76	28
7	2.2	—	60	104	59	56	48
8	2.0	—	44	64	74	38	18
Mean value	2.3	—	65	103	97	61	44
9	2.0	450	134	128	173	110	80
10	2.0	—	127	89	221	194	117
11	2.1	—	0	50	88	187	133
12	1.9	—	87	72	75	125	62
Mean value	2.0	—	87	85	139	154	98

Assay of Metabolites of Taurinophenetidine in Urine—A mixture of 1 ml. of 5 *N* HCl and 100 mg. of the frozen evaporate of urine of rabbits receiving taurinophenetidine (450 mg./kg.) was stored in an incubator at 37 ± 2° for 1 hr. After an addition of 1 ml. of 5 *N* Na_2CO_3 solution, 30 μl. of the centrifugation supernatant was submitted to TLC. The spots corresponding to taurinophenetidine, *p*-acetamidophenol, *p*-aminophenol, and *p*-phenetidine were each scratched off the glass plate of the TLC, using a mixed solvent of benzene and $(CH_3)_2CO$ (2:1), and extracted with 3 ml. of water on a boiling water bath for 3 min. Only in the case of *p*-phenetidine, 3 ml. of EtOH instead of 3 ml. of H_2O was used for extraction at 37° for 10 min. After centrifugation, 1 ml. of the supernatant was used for assay of the corresponding metabolite.

Taurinophenetidine and *p*-phenetidine were assayed by the modified Folin method. *p*-Aminophenol and *p*-acetamidophenol were determined as follows: 1 ml. of the supernatant as mentioned above was mixed with 0.6 ml. of 0.4% potassium ferricyanide solution, and after 5 min., 0.6 ml. of 1% $Fe(NO_3)_3$ in 0.7 *N* HNO_3 was added. After standing for 5 min., 3 ml. of H_2O was added to the mixture and the absorbance of the solution was measured at 720 mμ after 5 min.

The calibration curves of the four compounds were prepared separately by TLC as mentioned above, through spotting the solution of pure corresponding compound of known concentration.

Notes on determination of *p*-acetamidophenol—*p*-Acetamidophenol in the metabolites of taurinophenetidine in urine must be hydrolyzed by treatment with 5 *N* HCl at 37° for 1 hr. In this case, the ratio of hydrolysis was obtained in the following manner. A mixed solution of 400 mg. of *p*-acetamidophenol and 5 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr., 5 ml. of 5 *N* Na_2CO_3 solution and appropriate amount of H_2O was added to bring the total volume to 50 ml. Five microliters of this solution was submitted to TLC, using a mixed solvent of benzene and $(CH_3)_2CO$ (2:1). As a result, it was ascertained that about 11.3% of *p*-acetamidophenol was hydrolyzed to *p*-aminophenol. From this point, the ratio of hydrolysis of *p*-acetamidophenol must be considered when the ratio of *p*-acetamidophenol to the total metabolites in urine is calculated. The contents of four metabolites in urine were 117 mcg. of *p*-aminophenol, 623 mcg. of *p*-acetamidophenol, 202 mcg. of *p*-phenetidine, and 2.198 mg. of taurinophenetidine in 100 mg. of the frozen evaporate of rabbit urine.

Glucuronide in Urine of Rabbit given Taurinophenetidine—Four rabbits (2.5 kg. weight) were each given 0.7 g. of taurinophe-

netidine with H_2O by a stomach tube. In the next 5 hr., 350 ml. of urine collected was acidified with a few drops of glacial acetic acid and treated with saturated normal lead acetate until no further precipitate was formed. The filtrate was neutralized with NH_3 and treated with excess saturated basic lead acetate solution. The precipitate was centrifuged and washed twice by centrifugation with H_2O. The lead salt was suspended in H_2O and decomposed with H_2S. The filtrate separated from PbS was concentrated *in vacuo* at 45–50°. The glucuronide separated as a crystalline solid and was dried. The residue was shaken with EtOH and the solution was filtered. The glucuronide was then washed with EtOH and Et_2O (yield, 40 mg.).

The glucuronide was hydrolyzed with a small amount of 5 *N* HCl at 37 ± 2° for 1 hr., and neutralized with 5 *N* Na_2CO_3 solution. This solution was submitted to TLC: solvent, benzene and $(CH_3)_2CO$ (2:1); adsorbent, diatomite (Kieselgel G); color developer, 0.4% potassium ferricyanide solution and 1% $Fe(NO_3)_3$ in 0.7 *N* HNO_3.

Only *p*-aminophenol was detected at R_f 0.45 and identified by admixture with authentic sample.

Glucuronide in Urine of Rabbit Given p-Aminophenol—Four rabbits (2.5 kg. weight) were each given 0.7 g. of *p*-aminophenol with H_2O by a stomach tube. During the next 5 hr., 500 ml. of urine was collected and worked up as before to separate the glucuronide (yield, 450 mg.).

This glucuronide was hydrolyzed and submitted to TLC as before. When 5% $AgNO_3$ solution was sprayed, two spots of *p*-aminophenol (R_f 0.45) and *p*-acetamidophenol (R_f 0.35) were detected and identified by admixture with authentic samples.

RESULTS AND DISCUSSION

Before quantitative determination of taurinophenetidine in blood, it was ascertained that no metabolite of the chemical were observed in TLC. Taurinophenetidine seems to be absorbed with pseudo-first-order reaction by passive transport, since the peak of blood level is almost proportional to the dose when logarithm of blood level is plotted against time, from plasma data of taurinophenetidine shown in Table I and in Fig. 1.

The binding of taurinophenetidine with rabbit serum protein *in vivo* at different concentrations of blood level shown in Table I (at 350 mg./kg. dose) was examined but, actually, no binding of the drug with serum protein was observed within experimental errors. On the other hand, the binding ratio of taurinophenetidine with rabbit serum proteins *in vitro* was about 67%. It may be said positively that there is no distinct combination between taurinophenetidine and serum protein *in vivo*, unlike that *in vitro*.

Metabolites of taurinophenetidine in rabbit urine were studied on the frozen evaporated residue of the urine within 48 hr. after oral administration of 450 mg./kg. of the drug to four rabbits. When the frozen residue was submitted to TLC, four spots of *p*-phenetidine, *p*-aminophenol, *p*-acetamidophenol, and unchanged taurinophenetidine were separated and identified by admixture with the authentic samples.

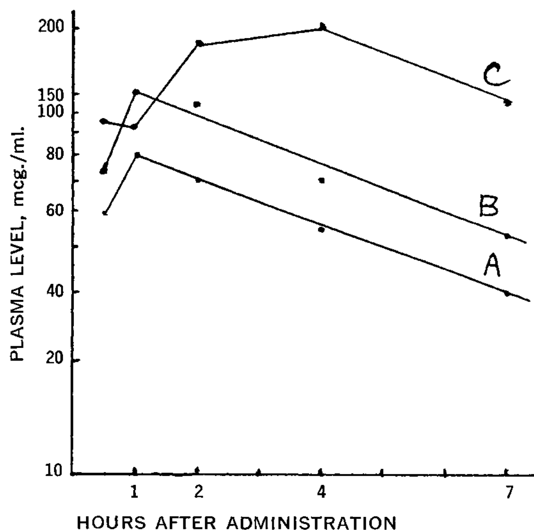
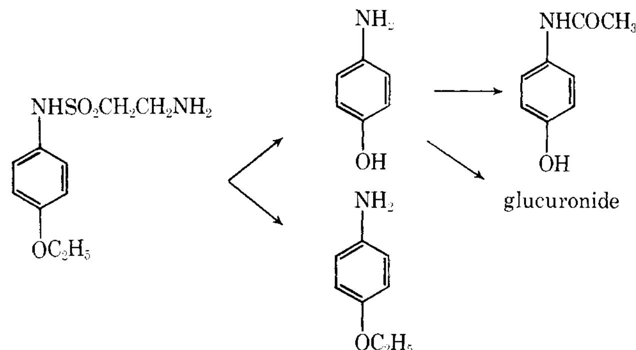


Figure 1—*Mean plasma level of taurinophenetidine after oral administraton to rabbits at different doses. Key:* A, *250 mg./kg.;* B, *350 mg./kg.;* C, *450 mg./kg.*

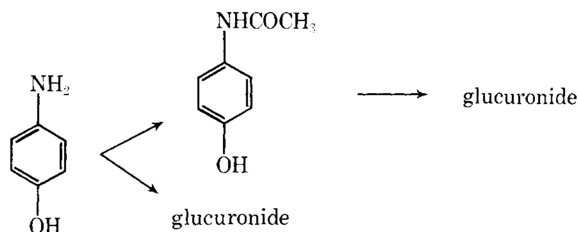
The results of quantitative determination of the metabolites in urine suggested that 3.7% of *p*-aminophenol, 19.8% of *p*-acetamidophenol, 6.4% of *p*-phenetidine, and 70.0% of the unchanged taurinophenetidine of the total metabolites were excreted. The ratio of the total metabolites to the dose ingested could not be calculated because of incomplete urine collection. The ratio in man will be determined later.

It was also identified by TLC with authentic samples that only *p*-aminophenol in the metabolites is excreted in the form of glucuronide from the glucuronide separated by the method of Williams (3).

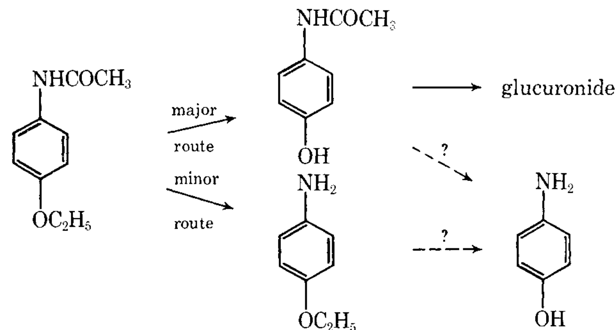
Many workers have already reported the metabolism of acetophenetidine which has a similar chemical structure to taurinophenetidine. According to Brodie and Axelrod (4), the analgesic and antipyretic effects of acetophenetidine are probably due to the formation of acetamidophenol as in the case of acetanilide. They also reported that about 84% of the dose was excreted during 24 hr., as *p*-acetamidophenol in man. The distinct difference between acetophenetidine and taurinophenetidine was in their main metabolites, such as *p*-acetamidophenol in the former and unchanged taurinophenetidine in the latter. The metabolic pathway of taurinophenetidine in rabbit urine will be as follows:



When *p*-aminophenol was administered orally to rabbits, as in the case of taurinophenetidine, both the glucuronides of *p*-aminophenol and *p*-acetamidophenol were separated by TLC;



In the case of acetophenetidine, the following metabolic pathway was indicated by Brodie and Axelrod (4):



A trace of unchanged *p*-acetophenetidine, 68.3% of *p*-acetamidophenol, 26.0% of *p*-aminophenol, and 5.7% of *p*-phenetidine of the total metabolites were obtained by the authors after reexamination of the metabolites of *p*-acetophenetidine, using the same procedure

as in the case of the metabolites of taurinophenetidine in rabbit urine.

In conclusion, it was ascertained that the main metabolite of taurinophenetidine is the unchanged taurinophenetidine which is different from the main metabolite of acetophenetidine, that is, *p*-acetamidophenol. Nevertheless, the structure of taurinophenetidine is similar to that of acetophenetidine. Therefore, it became possible to predict that taurinophenetidine might have characteristic pharmacological action comparing with acetophenetidine. From the results of the basic experiments in the present work, biopharmaceutical and pharmacological characteristics of taurinophenetidine will be discussed in the next paper.

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Behavior of Erythrocytes in Various Solvent Systems V: Water-Liquid Amides

DONALD E. CADWALLADER and JANIS R. PHILLIPS

Abstract ☐ Hemolytic behavior of human erythrocytes in water-amide solutions was investigated. Complete hemolysis of erythrocytes occurred in all formamide, DMF, DMA, DEA, and DEF solutions. Sodium chloride was effective in preventing complete hemolysis in solutions containing up to 10% formamide, 20% DMF, 20% DMA, 8% DEA, and 0.2% DEF. The addition of sodium chloride to solutions containing more than the above concentrations of amide did not prevent hemolysis, discoloration, and/or precipitation of human red blood cells. The addition of isotonic phosphate buffer (pH 7) to solutions increased the critical concentration at which the amide solutions damaged red cells. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in the various amide solutions.

Keyphrases ☐ Erythrocytes behavior—water-amide solutions ☐ Hemolysis, erythrocytes—water-amide solutions ☐ Sodium chloride effect—erythrocyte hemolysis ☐ Phosphate buffer effect—erythrocyte hemolysis

It is well known that to prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. For this reason, investigations have been made to study the hemolytic effects of aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols on rabbit and human erythrocytes and hemolytic effects of aqueous dimethylsulfoxide on human erythrocytes (1–4).

This report is concerned with the investigation of various water-liquid amide systems. The amides included in this investigation are formamide, dimethylformamide (DMF), dimethylacetamide (DMA), diethylactamide (DEA), and diethylformamide (DEF), Knazko (5) reported that pure formamide and dimethylformamide are satisfactory solvents for certain drugs, preferable for those which are hydrophobic and have an alicyclic structure and less favorable for compounds with heterocyclic structures. Very good solubility in DMF was shown by menthol, camphor, phenols, and sulfonamides. The solubility of sulfadiazine in water-DMF mixtures has been investigated (6). Spiegel and Noseworthy (7) in their extensive article on nonaqueous solvents for use in parenteral products surveyed the physical properties, toxicities, and parenteral applications of dimethylacetamide. They found this solvent to be miscible in all proportions with water and alcohol and very soluble in organic solvents and mineral oil. Waaler (8) demonstrated that DMA is extremely stable in 1 *M* aqueous solutions. For instance, the decomposition at pH 4.2 was about 1% after 600 hr. A study of the acute toxicities of DMA, DMF, and propylene glycol was made by Davis and Jenner (9) after single doses were administered intraperitoneally to mice. Results were as follows: LD_{50} for DMF was 1122 mg./kg., for DMA 3236 mg./kg., and propylene glycol 11,400 mg./kg. The LD_{100} for DMA was 5012 mg./kg.

The purpose of this investigation was to observe the behavior of human erythrocytes in aqueous formamide, DMF, DMA, DEA, and DEF solutions. In each experiment, the hemolytic method was utilized. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride-water-amide solutions, it was possible to calculate hemolytic isotonic coefficients for sodium chloride in various water-amide solutions.

EXPERIMENTAL

Materials—Formamide, purified, and reagent grade dimethylformamide (J. T. Baker Chemical Co.) and reagent grade dimethylacetamide, diethylformamide, and diethylacetamide (Eastman