ISOLATION, DETERMINATION AND DIFFERENTIATION OF MORPHINE, CODEINE AND HEROIN FROM VISCERA AND BODY FLUIDS BY CHROMATOGRAPHIC ANALYSIS

THESIS

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I. INTRODUCTION

None of the great number of techniques used for the quantitative determination of morphine and related alkaloids seems to have attained universal favor, probably because of the difficulty in obtaining concordant results with any of them. Most of the methods reported in the literature are even moderately satisfactory only when the amounts of alkaloids in the tissues or tissue fluids are relatively high, as in cases of acute poisoning or in experimental animals receiving a high alkaloid intake. With existing procedures it is probably too much to hope for the development of a method of isolation of the alkaloids as reliable as those for inorganic substances.

From the very earliest elaborated method for the isolation of alkaloids to its most recent modification, the detection of alkaloids in animal tissues and fluids, and in particular morphine, has involved complicated and time-consuming extraction procedures, and purification processes which are limited, to a high degree, by uncertainty.

The processes at present in use for the separation of alkaloids from complex organic mixtures depend on ultimate extraction into solvents immiscible with water. A method which will give good results for a certain tissue or excretion may not necessarily be as accurate for another. The various methods proposed for extraction by immiscible solvents are variations and improvements upon the method first proposed by Stas. His method

was the earliest systematic procedure developed for the isolation of the alkaloids. It was founded on the observation that the alkaloids form acid salts which are soluble in water and alcohol, but that the alkaloidal bases liberated by alkali pass more or less completely and readily into ether or other immiscible organic solvents.

Stas recognized the limitations of his method for purity in the isolation of the sought alkaloid, since he cautioned against the use of basic lead acetate or charcoal for purification purposes. The use of lead acetate for removal of foreign materials fell short of its purpose since it did not completely remove the foreign substances. He expressed himself even more strongly against the use of hydrogen sulfide that had to be passed through the liquid to remove excess lead since it entered into combination with many organic substances which later underwent decomposition on exposure to air, or on application of heat and thereby caused the liquid to become colored. Animal charcoal which could be used to decolorize the fluid under examination certainly did this, but removed the alkaloid as well.

Shortly after the appearance of the Stas method, modifications for its improvement were added and further refinements in the method have continued up to the present time. The first major modification suggested by Otto for the preliminary purification of the alkaloid, consisted in agitating the final aqueous liquid, while still acid, with ether for the purpose of removing fats and other substances (other than the alkaloids) soluble in ether. Another modification applied by Otto was the use of amyl alcohol in place of ether for the extraction of morphine.

These modifications by Otto led to the classical Stas-Otto method. Each new modifications, whether it was a new step for the treatment of the tissue or other material, a renewed effort for the purification of the alkaloid, or a substitution of one organic solvent for another, added another name to the Stas method. At one time a process embodying certain modifications which were more advantageous than the preceding ones might have been described as the Stas-Erdmann-Uslar-Otto-Dragendorff-Marmé method. In spite of the imposing list of modifications the essentials of the process remain basically those of Stas. The principle underlying the Stas method plus its modifications are simple enough but, as Bamford pointed out, the actual technique of obtaining the alkaloids in the state of purity which is required for absolute identification can only be acquired by experience and almost infinite patience. Meticulous attention to details is absolutely necessary.

The extraction of the alkaloids then, presents serious difficulties. The number of methods that have been proposed is legion and the very number is excellent evidence that they are unsatisfactory. Not one of these has received successful employment without modification by a considerable number of investigators.

The first part of this thesis consists of a review of these modifications insofar as they have been applied to the special case of morphine. The purpose of reviewing the literature is twofold. First, to emphasize the very serious and outstanding defects of as many methods for determining morphine, codeine, and heroin as possible and to ascertain whether the reported methods are sensitive enough for the work undertaken. Second, to deter-

mine what is known of the fate of morphine in the organism since this is important in a toxicological analysis. No attempt is made to review the literature completely but efforts have been concentrated on presenting the important findings and conflicting results. Experimental quantitative morphine recoveries only are considered; consequently nearly all findings prior to 1890, the year in which the first quantitative method was developed, are excluded from this review.

What the organism does to the alkaloids, morphine specifically, remains partially obscure in spite of many attempts at its elucidation. Once the morphine has entered the organism it has never been recovered in its entirety again nor has the lost fraction been completely accounted for. Several unsuspected sources of morphine loss have been revealed in recent years. Some of the morphine is eliminated unchanged in the urine and feces but some also is excreted in an undetermined conjugated form. Some was isolated from various tissues, although other tissues, unsuspected, contained proportionately larger amounts. Still another considerable portion of the morphine escaped recovery through conversion to an oxidized form which seems to have been neglected or considered to be negligible because of the incapability of the methods to isolate them. The primary obstacle to the solution of this problem of morphine disposal is the lack of an adequate method of estimating the concentration of morphine, as such, in the tissues and excretory products. An adequate method must be delicate enough to deal with minute amounts and be specific enough to measure consistently, known small quantities of morphine, codeine and

heroin.

The various methods which have been proposed can be classified into three groups: isolation from tissues, isolation from blood and isolation from urine. Each main group required further sub-division to evaluate the numberous steps in the extraction. The extraction of the alkaloid from the tissues and fluids involves as a first atage, the maceration of tissue; this is followed by preliminary extraction of the alkaloid from tissue pulp and excreta; from the extraction there must be a removal of soluble proteins, fat and other lipoids, coloring matter, and other impurities; this step is succeeded by the isolation of the alkaloids in a crude form; purification of the alkaloids is then necessary before its final isolation; and eventually the purified isolated alkaloids must be identified and quantitatively determined.

This review is followed by an account of a method as evolved by the author, with an appraisal of its value. The method consistently recovered 1 mg. or less of morphine, codeine and heroin from tissues, blood and urine, and it has been further developed to differentiate possible mixtures of the 3 alkaloids.

Finally, a short section records preliminary experiments which have been made with the object of determing whether the adsorption method proposed for morphine and related alkaloids may be extended to become a general means of searching for organic poisons.

Part I.

REVIEW

ISOLATION OF ALKALOIDS

QUANTITATIVE DETERMINATION OF MORPHINE

FATE OF MORPHINE IN THE ORGANISM

PSEUDOMORPHINE

CODEINE

HEROIN

II. ISOLATION OF ALKALOIDS

A. Maceration of Tissue

Most of the descriptions of methods simply indicate that the organs are finely pulverized, whether it be by a mortar, scissors, meat chopper or by some other mechanical means of reducing the organs to the finest possible state of disintegration. Nothing definite appears on this point. In experiments involving the addition of alkaloid to tissue or tissue mass, the degree of maceration is of little importance as there is no penetration of the tissue cells by the alkaloid, but for the alkaloid present within the tissue cell itself the degree of disintegration assumes major importance. The finer the mincing the smaller is the loss of alkaloid. FRENKEL (1910), BALLS (1926) and ZANELLA (1932) proceeded by disintegrating the tissue with the aid of quartz sand. Zanella carried the process even further and autoclaved the macerated acidified material, then completely dried the mass and finally pulverized the dried mass. PLANT and PIERCE (1933) reduced the tissue to a uniform creamy consistency in a mortar with the aid of glass powder. DAUBNEY and NICKOLLS (1937) found it impossible to achieve satisfactory mincing of the tissue with the ordinary maceration procedures. To get complete rupture of the tissue cells they froze the tissue in the refrigerator over-night. The material was then ground up in the solid state which resulted in a fine state of division. FABRE (1925) first utilized the enzymatic digestion of the tissue proteins.

He obtained a perfectly limpid fluid on digestion with pancreatine (trypsin). TERUUCHI and KAI (1927), KABASAWA (1934) and ENDŌ and KATŌ (1938) utilized proteolysis by papain digestion. GONNERMAN (1906) and FABRE (1924) demonstrated that morphine was not altered during the course of pepsin, pancreatine or trypsin proteolysis.

B. Preliminary Extraction of Alkaloids

1. FROM TISSUE PULP

a. Alcoholic Extraction

The extraction of the alkaloid from the minced tissue presented serious difficulties. Most of the modifications that have been proposed for the improvement of the Stas-Otto procedure utilized solvents which precipitated the proteins and provided a liquid medium for the alkaloid and unfortunately a solvent for the organic impurities. STAS (1852) extracted the alkaloid from the minced tissue with alcohol, acidified with tartaric or oxalic acid and warmed to 21-24°C. The alcoholic filtrate was concentrated in a vacuum at a temperature of 35°C. With some investigators this method of tissue extraction still continued to find favor with very little modification. GOTTLIEB and STEPPUHN (1910) changed the acid to 0.5N hydrochloric acid and later TAKAYANAGI (1924) used hydrochloric acid and refluxed the mass. WACHTEL (1921) resorted to sulfuric acid and refluxing. RUBSAMEN (1908) inaugurated the use of absolute alcohol for this step. AUTENREITH (1928) used the same and refluxed for

a more complete extraction. The validity of this procedure is very doubtful as the tissue fluids dilute the absolute alcohol which can therefore produce no greater effect than 95 per cent. alcohol. BALLS and WOLFF (1928), by using absolute alcohol containing 0.5 per cent. hydrochloric acid, were able to recover up to 92 per cent. of morphine added to muscle tissue. When, however, they used alcohol with another mineral acid or with acetic acid, either 1 per cent. or glacial, they found that a coagulum was formed which was richer in morphine than the surrounding solution, and when part of this solution was analyzed a maximum of 79 per cent. of the added morphine was recovered. Methyl alcohol did not work as well in this extraction.

The most serious objection to the alcoholic extraction of the tissue arose from the fact that a quantity of the animal matter and fat, particularly phospholipoids and lecithin from the tissues were likewise soluble in alcohol. The solvents also carried along certain bodies which are designated resins and resemble the pigments and cholesterol. The residue left on the evaporation of the solvent was a bulky mass of these substances. In concentrating the different solutions to a small workable volume, BABEL (1904) suggested that the temperature should not rise above 70°C. since at this temperature a brown coloration appeared and insoluble materials were found which afterwards could not be successfully removed. DAUBNEY and NICKOLLS (1937) estimated that as much as 100 gm. of gummy residue might be obtained from the evaporation of the extract from 1 kilogram of tissue. The residue was sticky and largely insoluble in alcohol and yet had to be extracted with alcohol until freed of all its alkaloids. Loss might occur at each stage of the extraction with

alcohol. This made the purification of the alkaloids in the later stages very cumbersome.

b. Aqueous extraction

Various suggestions have been made for the shortening and simplification of this preliminary step by use of solvents other than alcohol for the preliminary extraction although alcohol has the great advantage of extracting practically all the alkaloids and the alcohol solution can be evaporated at low temperatures, not exceeding 35°C., within a reasonable time. DRAGENDORFF (1868) initiated the use of aqueous acids for the extraction of the alkaloids from the tissues, in contrast to the Stas procedure, which used alcohol to leach out the alkaloid, thus eliminating some of the fats and fatty constituents of the organic brei. Others following along this line substituted various acids for the original aqueous sulfuric acid extraction of Dragendorff. v.USLAR and ERDMANN (1861) used hydrochloric acid, CLOETTA (1903) used acetic acid and RISING and LYNN (1932) used tartaric acid for the aqueous extraction in order to avoid the action of the sulfuric acid, at an elevated temperature, on the less stable alkaloids recoverable in the general scheme of analysis. KAUZMANN (1868) discarded the hydrochloric acid method of extraction when he observed that later in the analysis it formed ammonium chloride which was quite insoluble in the organic solvent and was retained with the alkaloid in the final residue.

BABEL (1904), to prevent these inconveniences, utilized ammonium sulfate for the separation of the alkaloids from the tissue. A clear filtrate resulted. FLORENCE (1927)(1927a) substituted trichloracetic acid for ammonium sulfate since all the al-

kaloids, as trichloracetates, are sufficiently soluble to permit their separation from the coagulated tissue proteins. DAUBNEY and NICKOLLS (1937)(1938) were opposed to this method of extraction on the grounds that the resulting coagulated proteins had a rubber-like consistency which did not lend itself to proper washing of the precipitate and drainage of the fluids. They felt that the granular precipitate produced by the ammonium sulfate precipitation was less objectionable than the sticky mass of the trichloracetic acid precipitate. On the other hand, PLANT and PIERCE (1933) tried various methods of separation of the alkaloid from the tissue proteins, by digestion with enzymes, by extraction with organic solvents and by heating and saturation with neutral salts. They found that by treating a fresh finely divided aqueous suspension of the tissue with trichloracetic acid, a solution of the alkaloid was obtained that was easily handled and which gave consistent results in control experiments with added morphine.

Another method for protein precipitation was used by KEESER, OELKERS and RAETZ (1933). The macerated tissue was treated with a 29 per cent. uranyl nitrate solution. Although these types of extractions introduced a liquid medium, at the same time they produced an abundant precipitate. CHERAMY and PAPAVASSILIOU (1939) intimated that such a precipitate might contain part of the alkaloid. Their own contribution was a tartaric acid-acetone extraction of the pulped tissue.

2. FROM FECES

A number of investigators developed methods for the extrac-

tion of alkaloid from feces. An acid-alcohol extraction on dried feces appears to be the favored method. TAKAYANAGI (1924) and PIERCE and PLANT (1932) used this principle. BALLS and WOLFF (1928) recommended a method which was coupled with a preliminary extraction with acid-alcohol mixture and a fat separation. With firm, friable feces, they obtained satisfactory results but with diarrheal excreta or feces containing a large proportion of undigested food, the results for morphine recovery, although high, were very variable. A saturated sodium bicarbonate solution-extraction of dried feces was the procedure suggested by OBERST (1942).

C. Removal of Soluble Proteins

1. TISSUE PROTEINS

The leaching out of the alkaloids from the tissues by either the alcoholic or aqueous extraction removed quantities of proteins and peptones as well as lipoid material. STAS (1852) in his method, after the evaporation of the original alcoholic solution, then treated the residue with absolute alcohol which dissolved the alkaloid and left the greater part of the tissue residue undissolved. DRAGENDORFF (1868) used the same procedure as the protein break-down products are soluble in aqueous acid solution as well as alcoholic solution. KIPPENBERGER (1897) suggested the use of tannic acid for the precipitation of the proteins. Large amounts of tannic acid were required and the protein material as well as the alkaloids were precipitated as tannates. The alkaloid tannates, being soluble in glycerine, were

then separated from the protein tannates, which were insoluble in this solvent. GOTTLIEB and STEPPUHN (1910) preferred the removal of the proteins from the residue by heat coagulation after the addition of sodium chloride and acetic acid. A number of investigators resorted to dehydration of the proteins for their removal. HILGER and KÜSTER (1889) added gypsum and ELLINGER and SEEGER (1934) added sand to the concentrated filtrate and dried the whole mass while ZANELLA (1932) even desiccated the syrupy residue before extracting the alkaloids.

2. BLOOD PROTEINS

Most of the procedures devised for blood are similar to those used for the removal of the soluble residual tissue proteins. In practice, the details are varied with each kind of material. TAUBER (1890) acidified the diluted blood with acetic acid and heated to boiling to coagulate the proteins. A 4 per cent. trichloracetic acid was used by FLORENCE (1927a) to accomplish the same results. The protein precipitant, 3.5 per cent. uranyl nitrate was used by OELKERS, RAETZ and RINTELEN (1932). In both methods the coagulated proteins were removed by centrifugation and excess uranyl ion removed by precipitation with a sodium salt. MULL (1936) deproteinized the blood with 1 per cent. zinc sulfate and 0.5N sodium hydroxide. Alcohol precipitation of the blood proteins was incorporated in several methods. DECKERT (1936) used 4 volumes of 96 per cent. alcohol for 1 volume of blood and ENDŌ and KATŌ (1938) produced the desired effect by using 2 volumes of absolute alcohol. An improvement in the alcohol precipitation method was proposed by BALLS and WOLFF (1928).

They used 20 volumes of absolute alcohol containing 1 per cent. acetic acid and 3 to 5 grams of cetyl alcohol. The coagulum was broken up by a mechanical stirrer. The blood proteins precipitated by acetic acid-alcohol resulted in a retention of some of the morphine but the adsorption of the morphine was reduced to a minimum by the use of cetyl alcohol. The extraction of the morphine from blood by the acetic-acid alcohol mixture, according to these investigators, was not as efficient as that from muscle by a hydrochloric acid and alcohol mixture. This, however, could not be used for blood since it also dissolved large amounts of hemoglobin derivatives which interfered with the subsequent analysis. Complete removal of interfering substances, involving precipitation probably caused some loss of the substance sought. For the removal of proteins from cerebrospinal fluid, or aqueous humor, WALKER and WALKER (1933) used 20 per cent. sulfosalicylic acid. For fluids of high protein content, such as serum, they found it advisable to dilute the serum with 4 volumes of physiological saline before deproteinization in order to minimize the error resulting from adsorption of the alkaloid on the precipitated proteins.

3. URINARY PROTEINS

KEESER, OELKERS and RAETZ (1933) treated the urine with 4 per cent. uranyl acetate solution to remove the urinary proteins. Color and extractive material as well as various acids were removed with basic lead acetate by the NOTTA and LUGAN (1885) method.

4. ADSORPTION OF MORPHINE ON PRECIPITATED PROTEINS

Since it seemed very possible that a loss of morphine might occur through adsorption on the precipitated impurities and thus be a source of error in any of the methods using such a procedure, BALLS and WOLFF (1928) set about to determine the retention under reasonable conditions. In their estimation morphine was not adsorbed on proteins coagulated by heat and acetic acid. DAUBNEY and NICKOLLS (1938) found that finely divided precipitated proteins did not appreciably adsorb morphine from aqueous solution. WALKER and WALKER (1933) claimed that there was a marked tendency for morphine to adsorb on the precipitated proteins.

D. Removal of Fat, Lipoids and Coloring Matter

Occlusion of alkaloids in fat occurs to some extent but this loss need not be considered a serious source of error. For the removal of fat, soluble lipoids and coloring matter several steps were usually incorporated into each procedure depending upon the material treated. In the STAS (1852) method, an insoluble precipitate of fat obtained on evaporation of the alcohol was filtered. At the same stage of the procedure AUTENREITH (1928) obtained a considerable separation of fat and resinous matter upon the addition of water. DAUBNEY and NICKOLLS (1937) claimed that attempts to extract the alkaloid from solid or liquid fat with acidulated water did not give 100 per cent. yields. The fat had to be either (a) dissolved in petroleum ether and the alkaloid extracted with acidulated water or (b) dissolved in petroleum ether, acidulated water added and later the organic solvent removed by evaporation. If the residue was very fatty, BAMFORD (1938) found it

advantageous to avoid filtration at this stage. He removed the fat with petroleum ether after the aqueous phase was made distinctly acid, since he felt there was a strong possibility that the organic solvent would extract certain of the alkaloids if the aqueous solution was not sufficiently acid. BALLS and WOLFF (1928) agitated the aqueous acid concentrate with warm benzene and then added a high melting paraffin to the benzene. The paraffin on cooling collected the soluble fat into a cake which could then be easily removed. Most of the modifications for the removal of the soluble lipoids were more readily applicable to the aqueous residue either by direct concentration after extraction or by replacement of the leaching organic solvent with water. In the OTTO (1856)(1857) modification of the Stas method, constituting the Stas-Otto method, some of the lipoids were removed by ether from the aqueous-acid liquid. MARQUIS (1896) removed foreign matter which included glycosides (FLORENCE (1927a)) by extracting the aqueous acid solution, first, with cold and then with warm ether. As other solvents replaced ether as the solvent for morphine in the Stas-Otto procedure they were used in a similar way for purification. LANDSBERG (1880) and MARMÉ (1885) modified the Stas-Otto process, particularly for the extraction of morphine, by agitating the aqueous liquid while still acid with hot amyl alcohol. BONGERS (1894) utilized ethyl acetate for the identical purification. A series of solvents, mainly petroleum ether, benzene and chloroform, were used consecutively on the acid aqueous concentrate by DRAGENDORFF (1868). Although the last two reagents were used in this particular analysis to separate certain of the alkaloids in the classical

scheme of analysis, they undoubtedly removed interfering impurities. BALLS and WOLFF (1928) first determined the relative extractibility of morphine and other precipitable substances of urine with chloroform at different pH levels. At pH 4.0 they found that the extraction of interfering substances from the urine was rapid while the removal of morphine was practically zero. ZANELLA (1932) obtained a pure solution of morphine hydrochloride from tissue extract by purifying with benzene, twice with ether and twice with chloroform. DAUBNEY and NICKOLLS (1938) considered it unnecessary to remove traces of fat from the acid filtrate since fat remained in the first chloroform extraction.

The purification of the residue from evaporation of the preliminary tissue extract by dissolving in absolute alcohol, introduced by Stas, was also used by LANDSBERG (1880) and TAUBER (1890). The former treated the concentrated urine extract with absolute alcohol and obtained a resinous mass. The latter used the alcohol purification after lead salt precipitation. Salts and most of the amorphous organic, more or less colored, substances were separated. Petroleum ether alone was used and found sufficient when FRENKEL (1910) investigated morphine in tissues. BALLS (1926) used benzyl alcohol to dissolve lipoids. It served him excellently for tissues containing large quantities of lipoids, as brain, nerve tissue and adipose tissue. No alternative procedure had been found satisfactory. For cases with large amounts of fats or lipoids ZANELLA (1932) extracted with benzene prior to desiccation of the material for morphine extraction.

1. LOSS OF MORPHINE IN ACID EXTRACTION

As pointed out by BALLS (1926) it was generally not realized that butyl alcohol and alcohol containing chloroform extracted large amounts of morphine from even strongly acid solutions, at least if a continuous extractor was used. With butyl alcohol as a solvent he showed, that 72 per cent. of a 100 mg. morphine hydrochloride in 10 ml. solution was removed in 30 minutes with a continuous extractor. In a similar experiment, amyl alcohol removed 9 per cent. in the same time. The last traces of the alkaloid were removed only with difficulty. He concluded that in using such a procedure for purification, there seemed to be no possibility of basing an analytical process on this behavior. ELLINGER and SEEGER (1934) also indicated that the purification of a morphine hydrochloride solution by chloroform extraction signified a source of error, as small amounts of the salt were dissolved in the chloroform.

E. Elimination of Residual Impurities

In order to remove quantities of peptone-like substances which were not completely removed by the alcohol or other solvents, further purification steps beyond the classical Stas-Otto method were added to increase the purity of the final aqueous extract. One of the procedures which Stas cautioned against, namely, precipitation with lead salts, was utilized to accomplish this end. SONNENSCHEIN (1857) used a hot lead chloride solution on the aqueous acid tissue extract for removal of impurities. BRIEGER (1886) added an alcoholic solution of neutral lead acetate (sugar of lead) to the syrup from the alco-

holic extraction. TAUBER (1890) and CLOETTA (1903) duplicated the Sonnenschein technique but used basic lead acetate. Instead of adding the lead salt to the extraction liquid directly, Van ITALLI and STEENHAUER (1927) and BAMFORD (1938) concentrated the extraction fluid and then treated it with the lead salt. The excess lead was removed either by hydrogen sulfide or dilute sulfuric acid. The removal of the excess lead was essential. CLOETTA (1903) pointed out that the excess lead was soluble in alcohol and gave a precipitate in alkaline solution which could be mistaken for morphine. In addition to the lead precipitation BRIEGER (1886) used a mercuric chloride precipitation to remove ptomaines. For muscle and brain tissues, WACHTEL (1921) did not think that the clarification with lead acetate was sufficient. With these organs a second precipitation with copper sulfate was carried out on the aqueous concentrate obtained after the lead treatment. Excess copper was removed by hydrogen sulfide. KOBERT (1902) classified Kieselguhr (Terra silicea) as a decolorizing agent for alkaloidal solutions. Its use was limited to removal of small amounts of impurities.

1. ADSORPTION OF MORPHINE ON PRECIPITATED LEAD SALTS

By the precipitation methods the greater part of organic impurities could be successfully separated from the mixture. It seemed probable that a loss of alkaloid through adsorption on the precipitated impurities might be a fundamental error in all methods using such processes. WACHTEL (1921) claimed that the error was slight in the lead purification method if the lead precipitate was carefully washed. BALLS and WOLFF (1928)

found that this was not true of the precipitates formed by the basic lead acetate clarification of the alkaloidal solution. A lead precipitate from a tissue coagulum gave as much as 3.4 mg. of morphine when leached with alcohol. In a second experiment they recovered 2 mg. of morphine base from an alcohol treated lead precipitate which was obtained from a liter of water containing 0.3 gm. peptone and 150 mg. morphine. DAUBNEY and NICKOLLS (1937) considered such modifications to be dangerous in practice as protein precipitants are also alkaloidal precipitants. van ITALLIE and STEENHAUER (1927), on the contrary, recovered all of the 4 mg. of morphine added as a syrup (Sirupus Papaveris) to water treated with lead acetate. It had been repeatedly emphasized that with each precipitation the combined loss of alkaloid could be controlled by complete subsequent washing of all filtered residues and eventually by renewed leaching of the accumulated precipitates and purification of the combined wash fluids and original solution.

The consensus of opinion is that most of the methods of purification are unsatisfactory from the quantitative point of view. The more manipulations for purification the greater becomes the loss of morphine. Despite their inherent weaknesses all of the described modifications yield useful results. Complete removal of interfering substances involved repeated precipitations or extractions, each of which probably caused some loss of the substance sought. It is necessary that no loss of morphine should arise through the purification procedures. This was not the case in most of the modifications described and several investigators quoted limits of errors of their methods from 30 to 40 per cent.

F. Destruction of Morphine on Alkaline Treatment

Although some of the investigators introduced into their methods certain modifications to avoid existing errors, they left themselves open to criticism in at least one other respect; they had ignored the decomposition of morphine in neutral or alkaline solution, particularly on evaporation. Some of these methods which did include such an alkaline evaporation gave recoveries, however, suggesting no such error. It was later recognized that the morphine oxidation products still retained many of the morphine reactions.

In the v.USLAR and ERDMANN (1861) method the aqueous tissue extract was made alkaline with ammonia and concentrated to dryness. In the TAKAYANAGI (1924) method the impure alkaloidal residue was mixed with quartz sand, made alkaline with sodium bicarbonate and dried. DECKERT (1936a) alkalinized urine with sodium carbonate and brought it to the boiling point. The same objection was levelled against the STEWART, CHATTERJI and SMITH (1937) method for exposing the easily hydrolyzable alkaloids, adsorbed on kaolin, to the action of hot concentrated sodium carbonate. OBERST (1942) similarly soaked dried powdered feces with saturated sodium bicarbonate solution for an hour or more before carrying out his extraction.

G. Isolation of Alkaloids by Extraction

1. ETHER EXTRACTION

The greatest number of modifications applied to the Stas method has been made in the selection of various solvents which

were used in the extraction of the alkaloids in the operation for preliminary purification. The fundamental requirement of the STAS (1852) method, and later the STAS-OTTO (1856)(1857) method, was the utilization of a series of organic solvents immiscible with water to effect a separation of the alkaloids. In the original method the alkaloids were isolated from an aqueous alkaline solution with 4 to 5 times its volume of ether. For the extraction of most of the vegetable alkaloids the method of Stas was readily applicable but for others it was either partially or not at all successful when the alkaloids were present in a very complex organic mixture like tissue pulp. OTTO (1856)(1857) found ether to be a poor solvent for morphine and later WORMLEY (1867) pointed out, morphine required nearly 8000 times its weight of ether for solution. The quantity of this solvent necessary for the extraction of even small amounts of alkaloids was, therefore, so great that it dissolved so much foreign matter as to render the ether solution unfit for application of special alkaloidal tests. This difficulty was removed to some extent, as first suggested by POELLNITZ (1867), by quickly agitating the aqueous solution with ether and decanting this solvent before the morphine crystallized. VOGT (1875) emphasized the quick agitation with warm ether. The insufficiency of the ether extraction of the Stas-Otto method in the search for morphine in cases of poisoning was the critical point. In the simplification and improvement of the extraction methods, it was generally overlooked that in the later Stas-Otto method free morphine was extracted not with ether but with amyl alcohol.

VALSER(1863) demonstrated that in materials treated accord-

ing to the Stas method and the residue exhausted with ether and then ethyl acetate separately, the morphine was found in the ethyl acetate residue and all the other vegetable bases were present in the ether residue. To extract free morphine JÖRGENSEN (1910) used ether containing 1 to 1.5 per cent. alcohol because it removed less foreign matter than did the usual morphine solvents such as amyl alcohol, chloroform or ethyl acetate. A greater percentage of alcohol was to be avoided as too much of this solvent in the aqueous phase would have retarded or entirely prevented the passage of morphine into the ether. Such a mixture in Jörgensen's opinion was far inferior to the usual solvents and therefore the recommended number of extractions was increased to about 10. Though claimed to be a poor solvent for strychnine and morphine, ether has still been used in recent years. van ITALLIE and STEENHAUER (1927) employed a preliminary extraction followed by an extraction with chloroform containing 10 per cent. alcohol. A similar procedure was used by FLORENCE (1927a) and MORGAN (1937), the latter using it for recovery of 25 to 250 micrograms of morphine.

2. AMYL ALCOHOL EXTRACTION

DRAGENDORFF (1861), in his analysis, tried out a succession of solvents, the last being hot amyl alcohol. v. USLAR and ERDMANN (1861) reintroduced a method for the isolation of morphine based on the fact that free alkaloid bases are easily soluble in pure, especially hot, amyl alcohol, when the aqueous solution was alkaline. The same solvent was used by LANDSBERG (1880) and DONATH (1886). The early workers obtained evidence

showing that amyl alcohol proved to be the best solvent, since in it morphine base was most soluble. Especially important was the use of absolutely clean amyl alcohol. Its use was open to the objection that it also dissolved many other substances, including cadaveric alkaloids (WITTHAUS and BECKER (1896)) with equal facility. For amounts up to 10 mg., ELLINGER and SEEGER (1934) determined that 30 ml. were sufficient for complete extraction. GÉRARD, DELÉARDE and RICQUET (1905) used an ammonia saturated amyl alcohol for the isolation. On the other hand, amyl alcohol extraction of morphine was found by BALLS and WOLFF (1928) to be inferior to chloroform alcohol extraction because of the fictitiously high results attained in muscle analysis. NEVES SAMPAIO (1922) considered amyl alcohol an unsuitable solvent for the extraction of morphine from urine. In a singular manner this solvent was abandoned accordingly and chloroform mostly substituted.

3.CHLOROFORM EXTRACTION

Chloroform was used by DRAGENDORFF (1868) as part of the general scheme of alkaloidal analysis. Since morphine was only very slightly soluble in ether KOBERT (1902) recommended that chloroform be used as one of several solvents. The utilization of chloroform, in BABEL'S (1904) opinion, had the advantage that all the alkaloids could be determined by the same method. It was not always exact, as he found that a trace of coloring matter always accompanied the extract and the crystals of morphine appeared somewhat yellow. The extreme difficulty of extracting small amounts of morphine quantitatively served to ex-

plain the variation of the solvents and time of extraction. BABEL (1904) found that 4 extractions were sufficient to completely remove the morphine from aqueous alkaline solution. TAKAYANAGI (1924) refluxed the solution with chloroform 3 times with a 20 minute period for each. A 5 hour period of extraction in a continuous extractor was favored by HOTTA (1932) while IKESHIMA (1933) used a 3 hour extraction period for amounts up to 6 mg. of morphine. TO and RI (1936) continued it for about 24 hours. That a small amount of morphine remained even after a 24 hour extraction with chloroform was stated by von KAUFMANN-ASSER (1913), who considered this as one of the sources of error in the method.

The alkalinity of the aqueous medium was found to be an important factor. Sodium bicarbonate or ammonium hydroxide were the preferable alkalizing agents. That chloroform does not extract morphine from strongly alkaline solution was shown by HATCHER and GOLD (1929). TO (1935) further showed that the morphine in a weakly alkaline solution was precipitated, but dissolved again in presence of excess alkali. The fact that morphine is a very weak base with a phenolic group and that its salts are hydrolytically dissociated in very dilute solutions was recognized by RÜBSAMEN (1908). He showed that after repeated neutralization with alkali, the free morphine was easily and quantitatively taken up by chloroform from solutions with a dilution of as great as 1 in 4500.

4. ISOBUTYL ALCOHOL EXTRACTION

Extraction with isobutyl alcohol found favor among a number

of investigators. MARQUIS (1896) extracted with ethyl acetate as well as isobutyl alcohol for total extraction. For complete exhaustion of the alkaloid with isobutyl alcohol CLOETTA (1903) recommended 4 to 6 extractions. The fact that isobutyl alcohol is as good a solvent of morphine as amyl alcohol and is without its unpleasant odor, seemed to NAGELVOORT (1898) to justify its use.

5. ETHYL ACETATE EXTRACTION

Support for the use of ethyl acetate, as well as isobutyl alcohol for the extraction of the alkaloids, was given by a few of the later workers. MARQUIS (1896) and BONGERS (1894) were the early instigators. Later it was tested and preferred by PANSE (1932) for the carrying out of a series of analyses of purification, since he obtained a good melting point and was not burdened with any odor. He used it for a direct extraction of alkalinized urine, as did DECKERT (1936a), for the determination of quantities ranging between 30 to 40 micrograms. By extracting once only, 60 to 70 per cent. of the morphine present was removed. The percentage of extraction was so constant that Deckert found it permissible to adjust the obtained partial value of incomplete extraction by multiplication. The time thus saved counterbalanced the slightly enlarged limit of error of the full yield. OBERST (1938) devised a rapid method for the extraction of morphine in urine using 2 extractions with this solvent. In addition to morphine, large amounts of impurities were extracted by the ethyl acetate and later precipitated by the alkaloidal reagent employed. These impurities had to be

separated as far as possible to prevent them from masking the final morphine reaction. ROCHE LYNCH (1938) chose ethyl acetate as a solvent for morphine, for although it was not 100 per cent. efficient, it was selective in its action. For morphine, BAMFORD (1938) also chose ethyl acetate as the extracting solvent. It dissolved this base less readily than chloroform-alcohol but yielded a cleaner extract and since the extract had at this stage been reduced to a very small volume, the relatively low solubility of morphine in this solvent was of less importance. The number of extractions required for complete removal of morphine from urine was small. GROSS and THOMPSON (1940) used only three extractions with equal volumes of ethyl acetate.

6. CHLOROFORM-ALCOHOL EXTRACTION

The almost unsurmountable difficulty of purification of the alkaloids from the organs with amyl alcohol recommended by Stas-Otto led to the use of chloroform. By mixing another solvent with chloroform the extraction of the alkaloid was made easier and more certain of recovery. KIPPENBERGER (1897) employed a chloroform-alcohol mixture (9-1) to extract morphine after saturation of the aqueous residue with sodium chloride mixed with concentrated sodium carbonate. Using the same ratio of solvents van ITALLIE and STEENHAUER (1927) extracted morphine from the aqueous solution alkalinized with ammonium hydroxide. AUTENREITH (1928) suggested a constant boiling mixture of the two solvents. The last traces of morphine, according to IPSEN (1912), could be removed after the extraction with chloroform by further extraction, once or twice, with a chloroform-absolute alcohol

mixture (9.5-0.5). One hour's extraction with chloroform-alcohol (3-1 or 4-1) was ample to remove 25 mg. of morphine, (BALLS and WOLFF (1928)). Several other variations in the chloroform-alcohol ratio were reported by the following experimenters. ZANELLA (1932) used a 4 or 5 time extraction with 18 per cent. alcohol and 82 per cent. chloroform by weight; PIERCE and PLANT (1933) used a 2 to 1 mixture. OBERST (1942) also used a 3-1 chloroform-alcohol mixture. Chloroform-methyl alcohol was considered by BAMFORD (1938) to be a better solvent for alkaloids than chloroform-ethyl alcohol. FRENKEL (1910) found that free morphine was extracted from the aqueous phase most readily by 3 to 5 extractions of a 3 to 2 mixture of chloroform-isobutyl alcohol, and later ABE and UCHIDA (1934) advocated a 3 to 2 mixture of chloroform-butyl alcohol instead of pure chloroform. About the same period KEESER, OELKERS and RAETZ (1933) introduced a 3 to 1 chloroform-isopropyl alcohol mixture for this extraction. One of the geat difficulties in the determination of morphine by the extraction procedure is the separation from even simple contaminating agents such as sodium bicarbonate. RISING and LYNN (1932) found the solvent best suited for such an extraction was a chloroform-acetone mixture. Extraction with the cold mixture had to be repeated 15 to 20 times for complete removal of the morphine while hot extractions necessitated at least 7 attempts. A small portion of adventitious material was always extracted by the solvents used; this impaired the results if the quantity of alkaloid present was small. Constant reference has been made in the literature to this well recognized phenomenon.

H. Direct Extraction of Alkaloids from Urine

The removal of urinary pigments and bases are difficult and there is nothing to be gained by a preliminary alcohol extraction such as that used for tissue. A few of the older methods used this superfluous step. In the LANDSBERG (1880), van RIJN (1912) and von KAUFMANN-ASSER (1913) methods, the acidified urine residue was taken up in alcohol which was later evaporated. The existing methods are particularly imperfect when applied to urine. Most investigators accomplished the isolation of alkaloids from the alkalinized urine by a direct extraction with some organic solvent, usually ethyl acetate or chloroform. In the BALLS and WOLFF (1928) method the morphine was extracted at its iso-electric point (pH 9.0). Since morphine was not readily extracted at pH 9.0 by pure chloroform, they substituted chloroform-alcohol mixture in which it readily comes out.

The use of several extraction solvents for the isolation of alkaloids as, for example, DRAGENDORFF (1868) had worked out in his method of determination for various plant bases was avoided by many investigators (IPSEN (1912), KRATTER (1890)). This was to avoid a distribution of any detectable amounts of alkaloid among a number of solvents. One and the same base is capable of going into various solvents as has been indicated. The identification of the alkaloids then becomes extraordinarily difficult. The chief defect of all the extraction methods lies in the uncertainty of a complete isolation and purification of morphine.

I. Solubility of Morphine in Organic Solvents

1. SUITABILITY OF SOLVENTS

a. Single Solvents

The little investigated basic question of the quantities of solvent involved in the separation of morphine from aqueous solution by a series of important organic solvents was experimentally determined by some investigators. Only those solvents not miscible with water were considered. Among various organic solvents, TAKAYANAGI (1924) found only chloroform useful for his purpose; solubility determinations showed that in 10 ml. of chloroform 5.3 mg. of free morphine was soluble while the acid salt of the alkaloid was insoluble. To extract 5 mg. of morphine completely from aqueous solution alkalinized with sodium bicarbonate, CORPER and GAUSS (1921) determined that 8 extractions with hot chloroform were required. van ITALLIE and STEENHAUER (1927) set forth a set of relative values of morphine solubility in a number of solvents. Five minute extractions with 10 ml. of the solvents gave a solubility of 3.0 mg. of morphine in chloroform, 7.3 mg. in chloroform and 10 per cent. absolute alcohol, 13.4 mg. in amyl alcohol at 15°C. and 14.0 mg. in hot amyl alcohol.

b. Mixed Solvents

The values of the solubility of morphine in the various mixtures of solvents used for extraction purposes either were not recorded or were not determined by the investigators who used

such mixtures. Some solubility values for mixed solvents were eventually determined. ANNELER (1912) found 1.7 gm. morphine soluble in 100 ml. isobutyl alcohol-chloroform mixture (1:1 by volume) at 15°C. BAGGESGAARD-RASMUSSEN and REIMERS (1935) determined the solubility of morphine in various ratios of isopropyl alcohol-chloroform and absolute alcohol (99.35 per cent.)-chloroform at 20°C. The maximum solubility of morphine, 650 mg., in 100 gm. of the former mixture was obtained when the ratio was 3.5 to 6.5 isopropyl alcohol-chloroform and 2.29 gm. in 100 gm. of the latter mixture when the ratio was 3 to 7 alcohol-chloroform.

2. ISOELECTRIC EXTRACTIONS

MAUX (1904) and KOLTHOFF (1925) found the solubility of morphine in water to be 14.3 mg. per 100 ml. and 16.5 mg. per 100 ml. respectively. The minimum solubility was at pH. 8.95 as found by the latter investigator. BALLS (1926) extracting as much as 31.5 mg. of morphine from an aqueous solution at a pH 9.1 in a continuous extractor, recovered 100 per cent. by extracting 50 to 60 minutes with a chloroform-alcohol mixture, 40 to 60 minutes with amyl alcohol, and 40 minutes with butyl alcohol. A solubility determination of morphine in chloroform-isopropyl alcohol mixture (3 to 1 by volume) was made by BAGGESGAARD and SCHOU (1930). The solubility of morphine in 10 ml. of this mixed solvent was found to be 0.7855 grams per 100 ml. Extraction of 200 mg. of morphine in 20 ml. of alkaline water with 25 ml. of the mixture at pH 8.2 gave a 99.5 per cent. recovery in 4 extractions and a 100 per cent. extraction at pH 9.2.

DECKERT (1936)(1936a) was able to extract almost 100 per cent. of the morphine from alkaline aqueous solution in 3 extractions with ethyl formate, ethyl acetate, isobutyl alcohol, amyl alcohol or phenylethyl alcohol.

The extraction of morphine at its isoelectric point is rapid. The extraction of a morphine solution with chloroform with phenolphthalein in the aqueous phase suggested that the latter became weakly acid in RUBSAMEN'S (1908) method, and he countered this reaction by the further addition of alkali. GOTTLIEB and STEPPUHN (1910), however, observed that the chloroform took up the phenolphthalein and the aqueous phase was therefore decolorized, which could mislead one into adding an excess of alkali. The early workers did not pay sufficient attention to this reaction, as the use of excess ammonia or other alkali held back morphine because the solution was too alkaline. In the opinion of TERUUCHI and KAI (1927) if the aqueous solution was less than pH 8.3 or if it exceeded pH 8.5 the recovery of morphine was diminished. They extracted 6 times with chloroform with frequent checks on the pH. Their work covered an extremely narrow range of pH. KABASAWA (1934), using a slight modification of the latter method, extracted the morphine in a continuous extractor at pH 8.3 for 3 hours with chloroform-ethyl alcohol mixture. No check was reported of pH changes during the operation. Notwithstanding the claims of Teruuchi and Kai that above pH 8.5 the loss of morphine was increased, BALLS (1926) and BALLS and WOLFF (1928) asserted that morphine was most easily extracted at its isoelectric point, pH 8.9. Up to pH 9.1 was allowable, but the aqueous solution retained con-

siderable quantities of morphine if the variation became larger, especially with amyl alcohol which is one of the best solvents for alkaloidal bases. They concurred with Terruchi and Kai that a variation on the acid side was of less importance with the lower alcohols and chloroform as solvents, due to the solubility of morphine salts in the alcohol and the practice of mixing the latter with chloroform. According to their investigation the necessity at this stage for a heavily buffered solution such as phosphates soon became apparent. WOLFF, RIEGEL and FRY (1933) extracted morphine from alcoholic extracts of feces with chloroform-alcohol solvent, after adjustment of the solution to the isoelectric point of morphine. BAGGESGAARD-RASMUSSEN and SCHOU (1930), as previously stated, brought out a subtle distinction between these extractions at pH 8.2 or 9.1. Examining extractions at pH 8.2 with 10 ml. chloroform and isopropyl alcohol (3-1) of 20 ml. water containing 200 mg. of morphine, they showed that 1 extraction yielded a 65 per cent. recovery and with 2 extractions an average of 95 per cent. recovery was obtained. At pH 9.2, with the same solutions, 1 extraction rendered about an 82 per cent. recovery and 2 extractions a 97 per cent. recovery.

3. DEHYDRATION OF SOLVENTS

Extraction of the aqueous phase with an immiscible solvent does not result in an absolute separation of the two phases. To remove the last traces of water retained by the organic solvent a number of investigators, AUTENREITH (1928), KEESER, OELKERS and RAETZ (1933), and MORGAN (1937) dried the solvent with anhydrous sodium sulfate. The literature is lacking in data as to

whether treatment with this dehydrating salt was capable of removing detectable amounts of alkaloid from the solvents.

J. Isolation of Alkaloids by Precipitation

The purely extractive methods of isolating the alkaloids retained in solution after the slow, laborious and often difficult procedures including the multiple modifications based on repeated precipitations of proteins, fats and interfering substances of a similar nature, became the impetus for attempts at not only the shortening and simplification of the extractive process, but also for its eventual elimination. Chief among these have been the precipitation methods. With the precipitation methods the main amounts of organic and inorganic materials which are of no interest remain in solution while the alkaloids are precipitated. The difficulties of separating the alkaloids from the interfering organic material could be diminished when the precipitation was carried out in an aqueous acid solution so that the proteins, fats, etc. would be excluded. SONNENSCHEIN (1857), using this principle, precipitated the alkaloids from the aqueous acid filtrate concentrate with phosphomolybdic acid. The precipitate was filtered and the alkaloid-phosphomolybdate complex decomposed in alkaline solution with barium oxide. The freed alkaloid was then extracted with alcohol. PALM (1857) and later BRIEGER (1886) modified this method by first treating the aqueous acid filtrate with lead acetate. The filtrate from this procedure, after removal of excess lead, was apparently clear and contained neither glycosides nor coloring matter. The alkaloid was then precipitated according to the

Sonnenschein method. The same method was applied to the determination of morphine in serum and whole blood by FLEISCHMANN (1929)(1929a). This procedure, more than the others, depended on the precipitation of the alkaloid with phosphomolybdic acid under optimal conditions. The better known morphine precipitant reagents of which potassium mercuric iodide, gold chloride, iodine-potassium iodide, phosphomolybdic acid and phosphotungstic acid are the most sensitive, were not considered sensitive enough by DECKERT (1936)(1936a) but a sufficiently sensitive reaction was found in the combined precipitation with vanadium-molybdic acids. His method used the reagents both for clarification and precipitation. After extraction of the urine with an organic solvent, the organic solvent residue was treated with ammonium molybdate to precipitate interfering substances. Lastly, the addition of the vanadate ion brought about the precipitation of the morphine as the vanadium-molybdate complex.

K. Isolation of Alkaloids by Adsorption

In recent years another alternative to the purely extractive means of isolating the alkaloids has been the development of the adsorption method. In view of the difficulty of extracting alkaloids from the gum-like mass of proteins, glycogen, and lipoids and also the tedious nature of the filtrations and evaporations, this method offered a promising way to extract them direct from the extract of tissue. The literature gives no evidence of any systematic investigation having been made to determine which adsorbents have affinity for all or some of the alkaloids and under what conditions the adsorption was maximal

or minimal. Many adsorbents have been found useful in removing alkaloids from solution in organic solvents and several have been used in aqueous media. Adsorbents have the great advantage of permitting the handling of large volumes of material and are a convenient way in which concentration of the sought alkaloid can be accomplished. Activated charcoal has been used but never successfully developed. Although LASSAIGNE (1824) used animal charcoal with success to decolorize the alcoholic extracts of animal matter, his method was criticised for the reason that it partially adsorbed the alkaloid as well. As previously pointed out, STAS (1852) urged discretion in the use of charcoal because of its adsorptive power for the alkaloids as demonstrated by GRAHAM and HOFMANN (1852). They, by reversal of the technique, withdrew added strychnine from beer with charcoal. While activated carbon will readily remove alkaloids from aqueous solution under almost any condition, elution of the absorbed material proved too inefficient to sanction its general use in alkaloidal work. Since LLOYD's (1916) discovery of the affinity of Fuller's earth for alkaloids, the search towards finding an adsorbent, which would adsorb alkaloids quantitatively has been given renewed momentum. Fuller's earth itself was notably inefficient in adsorbing alkaloids. STEWART, CHATTERJI and SMITH (1937) carried this work further by adsorption with kaolin from a trichloracetic acid filtrate from tissue pulp. Five grams of the kaolin adsorbed 50 milligrams of strychnine from 25 ml. of solution after a few minutes shaking. The adsorbent was filtered and the alkaloid was removed by mixing with a sodium carbonate solution and extracting with chloroform in a Soxhlet. From 88

to 97 per cent. recovery was obtained with strychnine. With smaller quantities, a correspondingly smaller recovery was realized. For morphine 80 to 95 per cent. was recovered for 10 mg. amounts. Atropine gave usually less than 50 per cent., for though readily enough adsorbed by kaolin it was easily hydrolyzed during the process. The use of permutit, a synthetic siliceous zeolite, an adsorptive reagent for amines was suggested by WHITEHORN (1923). OBERST (1939) applied the method to the determination of morphine in urine. The urinary residue, after the evaporation of the organic solvents, was dissolved in water and then shaken several times with permutit. One gram of this adsorbent completely removed 1 mg. of morphine from an aqueous solution. Most of the work in the field is of a preliminary or speculative nature and has not been correlated to toxicological analysis involving alkaloids.

L. Isolation of Alkaloids by Dialysis

Among the early approaches to circumvent the Stas-Otto type of extractive procedure was the method of dialysis discovered by Graham. HARVEY (1863) applied this to the separation of strychnine from organic matter. Much organic matter besides strychnine was contained in the diffusate and he concluded that as small as the amount of interfering material was that passed through the parchment paper membrane, it was sufficient to obscure the ordinary chemical reactions. VOGT (1875) used the same technique for the separation of morphine from urine. Further attempts by KOBERT (1902) with the method of dialysis were disappointing. He tried a number of experiments and they were

never entirely successful, especially in regard to the purity of the diffused alkaloid. Even when the diffusate contained quite a notable quantity of the alkaloid, the amount of colloidal or amorphous matter also present frequently required as much work for its removal as from the original mixture by immiscible solvents. Furthermore, he noted that a minute quantity of the alkaloidal base was still present in the mixture, as a portion of alkaloid always failed to pass through the membrane. Such small amounts could escape detection entirely, even when the quantity present in the original mixture was sufficient to give satisfactory results by the extraction method.

Electrodialysis showed more promise but its field of application is limited. FABRE and OFICJALSKI (1938)(1938a) used a 2 and a 3 compartment apparatus. The duration of electrodialysis varied in general from 6 to 24 hours depending on the nature of the product treated. Two hours of dialysis was sufficient for a pure alkaloid solution in contrast to an extract, rich in lipoids, which required 24 hours. A 94 to 100 per cent. recovery of 2 mg. of strychnine from fresh liver and putrified tissue was obtained by their method. For pure morphine solution with 6 hours' dialysis, an 84.5 per cent. recovery was made. With cocaine only a 72 per cent. recovery and with atropine a 52.7 per cent. recovery was realized. In each case the longer the passage of current the smaller was the recovery.

M. Purification of Alkaloids after Isolation

In many of the methods a further step for purification was of necessity added due to the impurity of the morphine after

isolation. The procedures for purification in this step varied but little from the purification preliminary to isolation of the alkaloid. While some of the methods concentrated on this preliminary purification, the following methods concentrated on the terminal purification. v.USLAR and ERDMANN (1861), who were the first to apply the Stas method for the isolation of morphine, used the acid-amyl alcohol purification. MARQUIS (1885) used the same type of purification which he then followed with a crystallization of the morphine from a mixture of hot chloroform-petroleum ether (1-50). PLANT and PIERCE (1933) purified with an amyl alcohol-chloroform (1-2) mixture. CLOETTA (1903) dissolved the residue in a mixture of slightly warm absolute alcohol-chloroform-benzene (2-2-1 volume). The impure morphine isolated by DAUBNEY and NICKOLLS (1938) was purified, first, by solution in acetone and then in chloroform. The simplest method of purification given by BAMFORD (1938) was to leave the residue exposed to air for 24 hours. This caused some of the impurities to become insoluble in acidified water. For stubborn cases, in which pure alkaloidal extracts had not been obtained by any of the mentioned techniques, the residue was taken up in water acidified with hydrochloric acid. The alkaloid was then precipitated with one of the double iodides. The alkaloid , then liberated from the precipitate by hydrogen sulfide, was extracted with the usual solvent.

Sublimation of the impure residue was also tried. KEESER, and KEESER (1928) sublimed the chloroform extract of morphine at 180°C. and found that the simultaneously sublimed lipoid droplets interfered. After 1 to 2 hours' sublimation they obtained long

morphine crystals which, on continued sublimation, decomposed again into small crystal pieces. PANSE (1932) suggested fractional sublimation of the residue. For small amounts of morphine OBERST (1940), after the usual acid and alkaline extraction with alcohol-chloroform mixture, further purified it by means of adsorption on permutit and extraction in acid and alkaline solution.

N. Emulsion Formation

One of the most troublesome impediments in the extraction procedures is the formation of an emulsion which may separate very slowly. Its formation is prevalent in the presence of proteins and lipoids. Some authors simply disdained from mentioning it while others made it a noteworthy point and devised measures to break it up. In the case of brain which gave a cloudy liquid on proteolysis and held in colloidal suspension fatty material and lipoids, FABRE (1925) offered the following elaboration. The liquid was centrifuged, acidified slightly and agitated with a little chloroform to destroy the emulsion. After centrifugation a liquid was thus obtained which could be submitted to the action of the immiscible organic solvents without fear of further emulsion formation and without sensitive loss of alkaloid. In the extraction of the aqueous-alkaline solution with chloroform or chloroform-alcohol mixture an emulsion might form that would not separate. To remedy this, AUTENREITH (1928) advised the addition of a few drops of alcohol and the solution placed on a warm water bath with occasional shaking. DAUBNEY and NICKOLLS (1938) encountered the same difficulty on the extraction of the filtrate

from the ammonium sulfate tissue solution with chloroform. The persistent chloroform emulsion was filtered through a layer of sand on a Buckner funnel. In the removal of fat from the aqueous acid solution with petroleum ether, BAMFORD (1938) reported that there often was a tendency for slowly separating emulsions to form. Several expedients, which were not always successful, were adopted to break them down. He tried the alcohol method with agitation, and the addition of a strong solution of such salts as sodium chloride or sodium sulfate with agitation, and filtration through a sand layer. On the direct extraction of urine with ethyl acetate OBERST (1939) observed occasionally a urine which formed an emulsion with the solvent. This was broken up by filtering the solution through a dry filter paper.

III. QUANTITATIVE DETERMINATION OF MORPHINE

Morphine is an alkaloid easily characterized even when it is present in minute quantities. By reason of its phenolic nature, it is soluble in the alkalies and is precipitated by alkaline bicarbonates. The quantitative determination of the isolated morphine used by the various investigators was conditioned by the type of preceding isolation and quantities of morphine obtained. In common with all chemical substances, the first method of quantitative determination of morphine to be considered is that of weighing the pure isolated substance. While weighing is the most accurate method for reasonably large amounts, for extraordinarily small amounts of alkaloids, this method is usually excluded. The advantage of a weight determination is that, after purification and isolation of the base, which is requisite in all methods, the weight of the latter may be found by simple evaporation of the solvent in a weighed container and determination of the resulting increase in weight. The method is not affected by variable factors, such as the choice of a suitable indicator in volumetric analysis and which, in one manner or another, influence the end results unfavorably. Finally, the method is completely independent of the formula, i.e. the molecular weight of the alkaloid sought. The quantitative methods described in the literature are extraordinarily numerous and only those dealing directly with toxicological analysis will be considered. Many of the methods for the final estimation of morphine are satisfactory. They are not all equally applicable, nor are they equally independent of the small amounts of impurities which may accompany the isolated morphine. With very small

amounts of this base there appears to be no avenue of escape from colorimetric methods in spite of their unreliability. With larger amounts there is a variety of procedures: gravimetric, oxidative, and acidimetric. In the presence of impurities both the titration and oxidation methods appear to be uncertain. HERZIG (1921) tried to reach a critical opinion of the value of the individual quantitative methods, the majority of which were methods for opium extracts. He arranged them and laid them under a critical discussion. For toxicological analysis a revaluation is attempted.

A. Gravimetric Determinations

1. MORPHINE BASE

TAUBER (1890) was one of the first investigators to attempt the quantitative determination of morphine. He precipitated the morphine salt from aqueous solution with sodium bicarbonate as the free base. The precipitation of the base depended upon the temperature, the alkaloidal concentration and the speed of the reaction. The slower the reaction the more crystalline was the precipitate. A white precipitate was obtained, dried at 100°C. and weighed. BABEL (1904) crystallized morphine slowly from chloroform forming very beautiful crystals which were dried at 80°C. HOTTA (1932) separated morphine from petroleum ether placed in a refrigerator for 24 hours. The morphine was dried at 95°C. for 1 hour. Weighing the base was used by HATCHER and GOLD (1929) as the preliminary step to check the colorimetric determination following.

Beyond the fact that the gravimetric determination has been used, it need hardly be given any serious consideration. For reasonable quantities of alkaloid such as 100 to 200 mg. of morphine determined in the Tauber and Babel estimations, it was successfully employed. For amounts of morphine ranging from 10 to 20 mg. Hotta reported recoveries of 73 to 80 per cent. He claimed that the recoveries, though not large, were always constant and the method quite reliable in its reproductivity.

The disadvantage of a direct weighing of the alkaloid in the isolation and purification, especially from tissues, is obvious. The alkaloid can hardly be separated from the adherent impurities, even though Hotta unequivocally stated that the morphine isolated by his method was so pure that there was no danger of weighing other impurities as morphine by mistake. For small amounts other investigators did not find it pure enough to weigh. On the other hand, a loss through further purifications cannot be avoided. A considerable number of investigators recognized that the isolated base, after evaporation of the solvent, was still more or less colored and that it was clearly not soluble without a residue. In the TAUBER (1890) method there existed the possibility that calcium salts would go into the morphine-containing solution from which it was precipitated as calcium carbonate with the addition of sodium bicarbonate. Consequently the values always ran high.

2. MORPHINE SALTS

This method of determining alkaloids is one in which there is produced a significant increase in the weight of the alkaloid

combined with some compound. A number of well known reagents such as phosphomolybdic acid, potassium mercuric iodide, iodine-potassium iodide solution, picric acid, phosphotungstic acid, silico-tungstic acid, vanadomolybdic acid, picrolonic acid and 1 chloro-2:4 dinitro benzene give a precipitate with morphine. These reagents give a definite precipitate in dilute solution so that very small amounts of alkaloid can be determined. The advantage of their use is that these reagents have a very much greater molecular weight and the weight analysis is facilitated for small amounts of alkaloid. Furthermore, the alkaloidal precipitant-alkaloid complex is in most cases less soluble in most solvents than the precipitants of simpler nature, so that danger of loss by washing is diminished. The exact constitution of the precipitate is in some cases still questionable. A point not to be overlooked is that these reagents also give precipitates with the alkaloidal contaminants of an organic nature.

3. PHOSPHOMOLYBDATE

The principle of this precipitation depends on the fact that morphine, under certain conditions, forms with phosphomolybdic acid a water insoluble precipitate. TAKAYANAGI (1924) adapted this method from the one Embden worked out for the gravimetric determination of small amounts of phosphoric acid. For the precipitation of alkaloids it was found that the alkaloid-phosphomolybdate precipitated after the ammonium phosphomolybdate. Through the use of an artifact the formation of the latter could be removed. With the help of ammonium phosphomolybdate in hydrochloric acid solution in the presence of oxalic acid, morphine was

successfully precipitated from aqueous solution. The necessary amount of oxalic acid was empirically determined. Takayanagi found the precipitation rapid and complete in 15 minutes. The precipitate was filtered on a Gooch crucible and dried at 100°C. It had been established by many experiments that a certain amount of morphine used corresponded to a definite amount of precipitate, i.e., in a ratio of 1:1.974. One milligram of morphine phosphomolybdate corresponded to 0.566 mg. morphine hydrochloride (plus 3 molecules of water of crystallization). The exact composition of the precipitate is unknown but it possibly possessed the following composition, $H_3PO_4 + 12\ MoO_3 + 4C_{17}H_{19}NO_3$. He worked with quantities ranging from 30 to 60 mg. morphine. According to FLEISCHMANN (1929)(1929a) the method gave good results for the range of 5 to 100 mg. morphine. ELLINGER and SEEGER (1934) showed that this method was good only from 40 to 70 mg. morphine; within these limits the error was within 5 per cent. With smaller or larger amounts of morphine the variation went to 50 per cent. They determined that the concentration of the solution controlled the amount of morphine phosphomolybdate precipitated; the greater the dilution the less was the precipitate.

4. SILICOTUNGSTATE

The principle of this precipitation is similar to the phosphomolybdic acid precipitation. The use of silicotungstic acid to precipitate morphine afforded a check upon the purity of the material estimated as morphine. BALLS (1926) and BALLS and WOLFF (1938) used Bertrand's silicotungstic acid precipitation for the determination of morphine in tissues. By extraction at the iso-

electric point of morphine, pH 9.0, the former was able to separate morphine from its oxidation products. FRY, LIGHT, TORRANCE and WOLFF (1929) determined morphine in urine by extraction with chloroform-alcohol solvent, purification through further extractions and final precipitation with silicotungstic acid. Later WOLFF, RIEGEL and FRY (1933) used a continuous liquid-liquid extractor for the isolation of morphine from urine with repeated isoelectric extractions until the residue was pure enough for a silicotungstic acid precipitation. The morphine silicotungstate was either dried and weighed on a Gooch crucible or ignited to constant weight. On ignition at a low red heat dry morphine silicotungstate gave an oxide residue of 70.3 per cent. Any other basic substance which differed from morphine in equivalent weight gave either a larger or smaller oxide residue. The extent to which the final morphine extract was contaminated with other basic materials was indicated by the ignition residue of the silicotungstate. An oxide content amounting to 71 per cent. of the dried morphine silicotungstate denoted appreciable impurities. WOLFF, RIEGEL and FRY (1933) gave the maximum allowable range as 69.5 to 71.5 per cent. for precipitates from urinary extracts. Feces presented a more difficult problem with this method than urine.

The composition of the morphine silicotungstate, like its predecessor the phosphomolybdate, is still doubtful. BALLS and WOLFF (1928) have asserted that 1 molecule of silicotungstic acid reacted with 2 molecules of morphine instead of the 4 as stated by Bertrand. They also precipitated known amounts of morphine under varying concentrations of hydrochloric acid and

sodium chloride and found that there was a slight deviation from the normal composition of the morphine silicotungstate as the concentration of the hydrochloric acid was increased. WOLFF, RIEGEL and FRY (1933) claimed that excess acid or heat at any stage of the analysis gave high results, usually indicated by the ignition value of the silicotungstate. The chief advantage of the silicotungstate method is its rapidity, simplicity, the exclusion of oxidized morphine if precipitated at proper pH, and the excellent check obtained by igniting the precipitate.

Its chief disadvantage is its lack of sensitivity, at least 4 mg. or more morphine being needed for a reasonably accurate analysis. When the quantity of morphine in the final extract was less than 10 mg. it was estimated colorimetrically although the use of a colorimetric reaction for the final estimation of morphine is open to some question.

5. DITROPHENYL ETHER

NICHOLLS (1937) attempted the quantitative determination of morphine by precipitation as the 2:4 dinitrophenyl ether. The precipitation was complete when it was carried out in approximately 30 per cent. alcohol and a considerable amount of ammonium hydroxide was used. After standing for 18 hours the precipitate was filtered through a Gooch crucible and dried at 100°C. Excellent results were obtained for quantities of less than 1 mg. to 70 mg. morphine. Other phenolic alkaloids may give insoluble ethers with this reagent. This method has not been extensively used.

B. Titrimetric Determinations

Some of the titrimetric methods for the determination of morphine are combined precipitation and titration methods. A reagent added, in excess, to the alkaloid-containing solution precipitates the morphine and the amount of alkaloid is determined by a measured amount of reagent required to precipitate it. Included in this category are the iodometric methods with their several modifications.

1. IODINE-THIOSULFATE

Morphine forms an insoluble precipitate with the added iodine in acid solution which is added in excess; either the remaining iodine is back-titrated with thiosulfate or the hydrochloric acid of a measured amount is back-titrated. The latter technique was used by GORDIN (1899). To determine the amount of morphine in solution it was mixed with a measured amount of N/20 hydrochloric acid and then with continuous stirring an iodine-potassium iodide solution was added until no further precipitate formed. The liquid containing the liberated iodine, freed of the precipitate by filtering, was reduced with sodium thiosulfate solution. The remainder of the hydrochloric acid in solution was back-titrated with N/20 sodium hydroxide using phenolphthalein as the indicator. This gave the amount of acid combined with the morphine. TO and RI (1938) used this method for the determination of morphine in urine. From 10 mg. morphine added to urine they obtained a 91 to 93 per cent. recovery. For smaller amounts there was a correspondingly smaller recovery to no recovery for 5 mg.

In the method of IKESHIMA (1933) the morphine was determined iodometrically. Sufficient iodine solution was added to the acid morphine solution so that the excess iodine could be back-titrated with sodium thiosulfate. He further showed that the iodine number which combined with a molecule of morphine was constantly 2.55 when a certain iodine concentration was maintained (amount of iodine in 1 ml. should correspond to over 1-1.1 ml. N/200 sodium thiosulfate). KABASAWA (1934) was able to apply this method for very small amounts of morphine and still maintain these conditions. This he accomplished by adding 1 mg. morphine hydrochloride to each cubic centimeter of solution under investigation and then the solution was treated with an excess of iodine. Ikeshima used quantities of 5 mg. and 6 mg. obtaining recoveries of 94-100 per cent. from blood and tissues. Kabasawa obtained a 95 per cent. recovery for 5 mg. and from 85 to 90 per cent. recoveries for 0.5 to 1 mg. morphine added to tissues.

2. IODOEOSIN

A direct titration of the morphine in solution was accomplished by von KAUFMANN-ASSER (1913) after its extraction from urine. The aqueous morphine solution was titrated with an alcoholic iodoeosin solution. Of 6 to 60 mg. morphine added to urine, between 68 and 83 per cent. was recovered. OSHIKA (1919) applied the same method to urine. The following results were obtained; for 65 mg. morphine a 64 to 76 per cent. recovery and for 5 mg. no recovery.

An obvious defect of the iodine precipitation method is that the impurities are likewise precipitated.

3. PHOSPHOMOLYBDATE-SODIUM HYDROXIDE

A modification of the phosphomolybdate precipitation method was used by OELKERS, RAETZ and RINTELEN (1932). The unknown amount of alkaloid in the phosphomolybdate precipitate was determined by titration with excess sodium hydroxide and back-titration with hydrochloric acid. Calculation of the alkaloidal content was accomplished by multiplication of the amount of standard sodium hydroxide with a factor, which was determined for each alkaloid by a series of experiments. KEESER, OELKERS and RAETZ (1933), using 0.025 N sodium hydroxide, calculated this factor for morphine hydrochloride as 5 in the presence of 0.2 to 0.7 mg. in 5 ml. fluid. Below this concentration of morphine a factor of 10 had to be used and above this concentration (to 1.7 mg.) a factor of 2.5 was necessary, indicating that the amount of morphine-phosphomolybdate precipitate did not increase in proportion to the increase in morphine content.

4. POTASSIUM FERRICYANIDE-IODINE

Still another titration method was used by WACHTEL (1921). The morphine was isolated by precipitating it as the phosphotungstate and then liberated from the phosphotungstate by trituration with barium hydroxide. The morphine was then oxidized with potassium ferricyanide. By the iodometric titration of the excess potassium ferricyanide used for the oxidation the amount of morphine was ascertained.

This method had many disadvantages. If the alkalinity was too low, low results were obtained since the reaction did not go to completion. If too much alkali was added, the results were

high. Wachtel claimed that amounts from 10 to 20 mg. morphine could be found within 1 to 2 mg. For quantities less than 5 mg., too high values were obtained so that the method was unusable. For values greater than 50 mg. the error ranged from 10 to 20 per cent.

5. SODIUM HYDROXIDE

Alkaloids are bases and form characteristic salts with acids. A method based on this fact was used by RÜBSAMEN (1908) as a preliminary determination only. The morphine base in solution was completely bound to acid and to extract it by chloroform by gradual addition of dilute sodium hydroxide a point was reached at which no new acid was set free. He found the best indicator to be a mixture of phenolphthalein and malachite green since the yellow color of the tissue extract was less troublesome than in the case of phenolphthalein alone. The morphine residue was checked by another method.

6. BROMINE

HATCHER and HATCHER (1935) described a method for the quantitative estimation of small amounts of morphine by means of an aqueous solution of bromine. The method depended on the absorption of the bromine, the end point being the disappearance of the yellow color. Attention had to be paid to concentration, temperature, and rate of reaction. The rate of reaction of codeine sulfate, heroin hydrochloride and morphine sulfate was increased by sulfuric acid. The acceleration increased with the concentration of acid. Several modifications were devised to

give a good end point. In the first modification, chloroform was added after the reaction was complete since the free bromine was taken up by the chloroform and assumed a yellow tint. In the second modification, used for all cases of very low concentrations of alkaloid, when the chloroform did not afford satisfactory results, a fraction of a milligram of apomorphine was added. A trace of free bromine was indicated by a pink color appearing within 30 seconds.

C. Colorimetric Determinations

For the determination of small amounts of morphine the colorimetric method is the one of choice. The color reactions are of 2 classes; in the one many alkaloids give the same color and in the other, the color is given only by one of the alkaloids or a chemically active group of the alkaloid. Most of these reactions are supposedly due to the formation of a complex unstable chromogenic substance. Development of a satisfactory colorimetric method for the quantitative determination of morphine has been retarded because of a general lack of selectivity and sensitivity of known reagents for this particular alkaloid and because of the impermanence of color produced. Many of the reactions, in addition, are influenced to some degree by the temperature and pH. of the test solution and the presence of commonly occurring contaminants. This condition is not unusual because of the formation of the chromogenic substances.

1. MARQUIS REAGENT (Formaldehyde-sulfuric acid)

MAI and RATH (1906) used this reagent for a quantitative

method. One mg. morphine diluted to 4 ml. with the reagent gave an opaque violet blue color while with smaller amounts of alkaloid, a color which was still measurable was obtained. They claimed that their method was sensitive in a range of 0.03 to 1 mg. morphine per ml. According to HEIDUSCHKA and FAUL (1917) the blue color of morphine with the Marquis reagent could be used quantitatively within the concentration of 0.07 to 0.9 mg. morphine per ml. A colorimetric estimation of morphine in amounts of 0.04 to 50 mg. using this reagent was developed by CORPER and GAUSS (1921). They found that it had an extinction coefficient corresponding to about 0.003 mg. morphine. When this method was applied to morphine extracted from tissue, HATCHER and DAVIS (1926) claimed that a small amount of a substance, which gave a reddish tint with the reagent, was also extracted. This made accurate comparison with the standard impossible with transmitted light when only traces of morphine were present, but in such cases the color could be compared with the color of the standard by means of reflected light fairly satisfactorily. The tint did not influence the comparison when more than very small amounts of morphine were present.

This type of colorimetric reagent is non-specific and offers no direct indication of the purity of the morphine finally estimated.

2. IODIC ACID

In its salt solution morphine shows oxidative ability whereby it can be converted into pseudomorphine. If a morphine solution is mixed with iodic acid, the acid is reduced and the solu-

tion due to the liberation of iodine becomes yellow. This reaction was used by GEORGES and GASCARD (1906) for the colorimetric determination of morphine. The yellow or reddish yellow coloration produced after the introduction of the iodic acid was changed to a yellowish brown tint, more or less stable, by the addition of a slight excess of ammonium hydroxide. In the method without ammonia the color was complete after one-half minute and diminished after 15 minutes; the color developed only after 2 or 3 minutes in the ammonia technique. The best results were obtained with 0.2 to 2 mg. morphine per ml. HEIDUSCHKA and FAUL (1917) developed the same method and found that 5 minutes after the addition of 10 per cent. ammonium hydroxide the color intensity reached its maximum and held its intensity for 2 hours. Their minimum values were within the limits of 0.18 to 0.66 mg. morphine per ml. If interfering yellow substances were present from the organic mixture extracted, van ITALLIE and HARMSMA (1926) suggested that the liberated iodine be extracted with carbon disulfide or carbon tetrachloride and the color of the new solution be compared with the standard.

3. AMMONIUM IODOXYBENZOATE

EMERSON (1933) proposed a simple quantitative colorimetric assay of morphine based on the iodoxybenzoate reaction. The color developed by oxidation with this reagent was dependent upon the number of phenolic groups; those with one phenolic hydroxy group yielded yellow colored oxidation products. The iodoxybenzoate did not decompose the compound on which it acted, but merely revealed the presence of free phenolic hydroxy groups

by an oxidizing color reaction. The specificity of the iodoxybenzoate for phenolic groups was relative since certain muzzled phenolic compounds as heroin, which hydrolyze readily, also reacted, although at a much slower rate.

The use of trichloracetic acid filtrates or extraction of ammoniacal solutions of serum or urine with chloroform was suggested by Emerson. The amount of iodoxybenzoate used had no effect on the intensity of the color produced but the maximum intensity was reached in a shorter time when larger amounts were used. Excessive amounts caused troublesome precipitation. The method was found to be sensitive to less than 10 mg. morphine per ml. Estimations in serum and urine gave errors amounting to 7 and 5 per cent. respectively.

The Emerson method was later modified to give a more accurate quantitative method by MOODEY and EMERSON (1939), who found that the hydrogen ion concentration of the reacting solution affected the rate of color formation and color fading. The color formed and faded rapidly in acid solution. In alkaline solution the color development was slow but was much more stable. The optimum pH was 6.8. The optimum reaction time was found to be 1 hour when the final concentration of ammonium iodoxybenzoate was 1 per cent. This method has a decided advantage as fewer substances interfere with the iodoxybenzoate reaction than with the reagents of other methods.

4. PHOSPHOTUNGSTIC ACID

FLEISCHMANN (1929)(1929a) used phosphomolybdic acid for a quantitative determination. The method depended on the precipi-

tation of the morphine with phosphomolybdic acid under optimal conditions and then the development of the color with concentrated sulfuric acid. A blue violet color formed with a maximum intensity at 1 hour. He quantitatively determined from 0.02 to 2 mg. morphine in 5 ml. water. The error for values under 0.6 mg. was under 1 per cent., between 0.2 to 0.5 mg. morphine, 3 per cent. and for the lowest determinable amounts up to 10 per cent. As with all sulfuric acid color reagents, deproteinization was an important condition for this determination, as proteins and other impurities gave a dark brown color.

According to KEESER, OELKERS and RAETZ (1933) this colorimetric method for organs and excretions was in general too uncertain.

5. PHOSPHOTUNGSTIC-PHOSPHOMOLYBDIC ACIDS

A colorimetric procedure for the determination of morphine, based on the ability of its phenolic group to reduce phosphotungstic and phosphomolybdic ion to colored products of a lower valency was developed by MULL and OBERST (1936). Mull dissolved the morphine-phosphomolybdate precipitate with 2 per cent. ammonium hydroxide resulting in the formation of a blue color which persisted for 20 minutes. Excessive amounts of ammonium hydroxide or stronger ammonia solutions dissolved the precipitate more readily but caused fading of the color. With 0.01 mg. morphine a clear blue color was obtained. The color production was not porportional to the concentration of morphine. 0.005 mg. gave a distinct color and 0.002 mg. was definitely distinguishable from the blanks. Standards for color comparison were pre-

pared from whole blood. Mull's finding that addition of the morphine to the blood filtrate gave darker color standards than those from whole blood indicated that there was some loss of morphine with the precipitated proteins. The loss was greater with the more concentrated filtrates, such as a 1 to 5 dilution and less with a 1 to 20 dilution.

OBERST (1939) used the FOLIN-DENIS (1915) phenol reagent which was a mixture of phosphomolybdic and phosphotungstic acids. In a strongly alkaline solution an intense blue color developed which was stable for a day. He found the test very sensitive for small amounts of morphine. As little as 0.05 mg. of the alkaloid diluted to 50 ml. could be determined. Uric acid and phenols had to be completely removed from the urinary residues for they gave a similar color with the phenol reagent.

6. SILICOMOLYBDIC ACID

HOFMAN and POPOVICI (1935) developed a new colorimetric method for the determination of small quantities of morphine using silicomolybdic acid. Their method depended on the property that morphine possessed to reduce the silicomolybdic acid in an alkaline medium producing a blue color, the product of reduction of the acid. They claimed that the maximum color intensity was attained in 5 minutes and was stable for 6 hours. The advantage of this reagent was that it permitted the characterization of the morphine without its isolation and merely after previously eliminating other reducible substances. The work of VAN ARKEL (1937) did not support the time of the color development of the previous investigators. From calibation curves for 0 to 5 mg.

morphine he demonstrated that after 2 hours the color was less by 20 per cent. than after 15 minutes. The maximum color intensity was obtained in 15 to 20 minutes.

7. DIAZO-SULFANILIC ACID

The colorimetric determination of morphine with the diazonium compounds has gained popular favor. LAUTENSCHLAGER (1919) introduced the method based on the color development with commercial diazobenzenesulfonic acid which was later discarded in favor of the freshly prepared diazotized sulfanilic acid. With Lautenschlager's reagent the color developed immediately in alkaline solution, going from a deep red to bright red depending upon the concentration of the alkaloid. O n acidification with dilute acid the color turned orange. The reacting group of the morphine molecule was the phenol group since no color was obtained in related compounds in which this group was masked with a methyl or ethyl group. For quantitative estimations solutions of concentrations between 0.05 to 0.5 mg. morphine per ml. were most suitable.

AUTENREITH and QUANTENMEYER (1928) substituted freshly prepared diazotized sulfanilic acid freed of all nitrous acid, by the addition of urea to prevent any interference with the color formation. The preference for the diazonium method as pointed out by PIERCE and PLANT (1932) was the production of a brownish red color which was proportional to the amount of morphine present within a certain dilution range. The color remained permanent thus permitting accurate colorimetric determination of the morphine. Maximum color intensity was attained within 90 min-

utes and standing for 2 hours did not affect the readings. The color of amounts as small as 0.1 mg. was of sufficient depth and tint when diluted to 2 ml. to give accurate readings against appropriate standards. They found that the purification of the morphine after the extraction from tissues and body fluids required less manipulations than for gravimetric or titration estimations. The only impurities that interfered were those that were colored or gave a color with the diazo compound. A check on these impurities was made by running a blank analysis on the material being examined for the alkaloid. OBERST (1939) determined the morphine of urinary residues concluding that 1 mg. was about the minimum quantity which gave a satisfactory color by the diazo reaction when the final dilution was 10 ml. When he treated a residue from 50 ml. morphine-free urine with the diazo reagent he obtained a red color similar to that obtained with morphine. Apparently substances other than morphine were present in the urinary residue which gave a color reaction.

8. OTHER COLOR REAGENTS

A few other scattered colorimetric methods have been recorded but not extensively used by succeeding investigators; these include color development with nitric-sulfuric acids, hydroquinone, bromine, ferricyanide and sodium nitrate. CARLINFANTI (1915) used the nitric-sulfuric acids reaction. The presence of morphine was shown immediately by a characteristic blue-red coloration which was estimated colorimetrically. This is one of the usual sulfuric acid reactions. WALKER and WALKER (1933) devised a method applicable chiefly to the quantitation of traces

of alkaloid in aqueous solution. In their method the alkaloid was precipitated as the phosphomolybdate, dissolved in dilute sulfuric acid and the color developed by the addition of an aqueous solution of hydroquinone and a carbonate-sulfite solution, similar to that used in the Bell-Doisy method for phosphate determination. Prompt comparison of the color with that of standards of known and approximately equal morphine content was necessary. The analytical error was less than 6 per cent. if more than 0.1 mg. was present. With amounts smaller than 0.1 mg. the error increased with decreasing amounts up to 20 per cent. for 0.05 mg. morphine, which was about the limit of possible colorimetric reading. RIZZOTTI (1935) produced a colorimetric method based on the reducing property of morphine by virtue of its free phenolic group. Thus ferricyanide was reduced to ferrocyanide and by the addition of ferric sulfate a Berlin blue reaction resulted. Special conditions of temperature, concentration, and alkalinity were ascertained and the optimal conditions were established. With this method he succeeded in estimating 0.025 mg. morphine with a maximum error of 2 per cent.

D. Nephelometric Determination

A recent innovation for the quantitative estimation of traces of morphine was attempted with the aid of vanado-molybdic acid. The method is based on the principle that morphine can be determined nephelometrically by the formation of an insoluble microcrystalline morphine-vanado-molybdate complex. DECKERT (1936) was the first to attempt this method of determination for morphine. He obtained the maximum turbidity in all cases within

20 minutes . After 18 hours the turbidity curve was identical with the one obtained after 20 minutes. As little as 10 micrograms of alkaloid could be determined in this manner. ENDŌ and KATŌ (1937) agreed that for 10 micrograms or more of morphine this method was easily and rapidly applied. They noticed, though, that equal amounts of morphine on different days gave varying degrees of turbidity. On investigating the factors which influenced the degree of turbidity, they concluded that Deckert's statement on the attainment of maximum turbidity was correct only if the quantities were less than 40 micrograms. In morphine solutions of greater quantities the turbidity increased and reached a maximum in 2 hours and did not change much after that. The turbidity was found to decrease with an increase in temperature. When this method was used for the assay of morphine in biological materials, ENDŌ and KATŌ (1938) further found that the ratio of the recovered morphine to the added morphine was different according to the tissues or fluids used, but in the same material it was always constant. A ll the recovery curves obtained were rectilinear. OBERST (1938)(1939) found the degree of turbidity to be roughly proportional to the amount of morphine present in urine. From evidence obtained, he stated that the amount of morphine precipitated as the complex, depended on a number of factors, such as the volume of fluid in which it was precipitated, the acid concentration of the reactant solution, the amount of impurities still present and the temperature of the solution. Excess acid increased the solubility of the complex. The complex coalesced and precipitated more rapidly in the presence of excessive amounts of impurities. For amounts of morphine exceeding 0.25 mg.

unsatisfactory results were obtained. Amounts as low as 0.03 mg. morphine in 25 ml. urine could be detected satisfactorily. Occasionally Oberst encountered some difficulties with this procedure. A residue containing large amounts of impurities often produced a voluminous precipitate following the addition of the molybdate solution. Occasionally a blue green color developed in the solution while it was standing for the precipitate to form; this color was due to certain of the impurities having strong reducing properties. The addition of the vanadate solution to the blue-green filtrate did not produce a turbidity even when morphine was known to be present.

E. Biological Determination

1. SENSITIVITY

In instances where small amounts of alkaloids were to be identified and quantitative chemical methods of procedure proved inadequate, some other method was needed to overcome this difficulty; thus the biological proof method based on experiments of Straub and Hermann was developed.

STRAUB (1911) and his pupil, HERMANN (1912) described a biological reaction for morphine which they thought was specific for that alkaloid and could possibly be used for the quantitative determination of extremely small amounts. Hermann stated that after the injection of 0.005 to 15 mg. morphine into mice, the spine assumed a lordotic curvature, the hind legs became slightly spastic and the tail arched into an S-shape over the back. The reaction started from 2 to 15 minutes after the injection, the

longer time for the smaller dose. For 5 mg. the reaction lasted 20 hours and the duration fell regularly with decreasing doses so that with 0.005 mg. it lasted only 45 minutes. He concluded that a direct relationship existed between duration of reaction and injected amount of morphine. No adequate explanation of this phenomenon was advanced by these authors.

RASSERS (1916) found 0.02 mg. to be the smallest dose of morphine to give this reaction. In a series of experiments with 20 gm. mice, MAIER (1931) showed that all animals did not react to small doses under 0.06 mg. morphine. At 0.06 mg. all mice showed the characteristic tail position and with doses under 0.06 mg. the differences in the reaction-time were relatively less. The curve of dose/reaction time was rather irregular. By both determinations, duration of reaction and per cent. of positively reacting animals, the test showed a surprisingly exact biological analysis of the amount of morphine. With 0.06 mg. morphine, 100 per cent. of the animals responded with an average duration of reaction time of 165 minutes. In MUNCH's (1934) experiments the amount of morphine required to show a 100 per cent. positive mouse tail reaction was 2.3 times greater than Maier's figure (7 mg./Kg. as compared to 3 mg./Kg.). KEIL and KLUGE (1934) asserted that the tail phenomenon, according to the morphine injection, was translatable quantitatively, so that the amount could be determined to 0.012 mg. with an accuracy of 5 per cent. They set up 2 curves. The first curve (number of positive reactions plotted against amounts given) showed a direct relationship between the percentage of positive animals and the injected amounts. In the second curve there was also a direct relationship between

the duration time and morphine quantity. TERADA and HONDA (1933) found that the relationship of duration of the tail-raising action to the quantity of morphine injected was manifested by the formula $T = kM^p$, where T was the duration of tail-raising reaction, M was the amount of injected morphine and k and p were constants.

v. KAUFMANN-ASSER (1913) specified that prior to the experiment in each case, the normal position of the mouse's tail had to be proven. He agreed with Hermann that there was no exact lower limit for a maximal reaction, but that this value varied with the different batches of mice and was on the average between 0.03 and 0.001 mg. morphine. His conclusion was that the quantitative determination of morphine in urine by the biological method could not be used but it was useful as a qualitative method. RASSERS (1916) made the same suggestion that the biological test in forensic cases should only be used as a help in identification after the alkaloid had been isolated.

2. NON-SPECIFICITY

HERMANN (1912) found that papaverine, narcotine, narceine, nicotine, thebaine and dionine gave reactions similar to morphine but gave irregular results and only with large doses. Codeine gave the same reaction but with ten times the doses. Other materials as cocaine (3 mg.), caffeine (5 mg.), camphor (20 mg.), picrotoxine and tetanus toxin in very small doses, and most important of all, potassium cyanate in the same dose as morphine were found by RASSERS (1915)(1916) to elicit the Straub-Hermann effect. JENSEN and RUMRY (1918) confirmed Hermann's finding that nicotine caused a similar reaction. While they found some differ-

longer time for the smaller dose. For 5 mg. the reaction lasted 20 hours and the duration fell regularly with decreasing doses so that with 0.005 mg. it lasted only 45 minutes. He concluded that a direct relationship existed between duration of reaction and injected amount of morphine. No adequate explanation of this phenomenon was advanced by these authors.

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properties was due to this substance which was related to some of the oxidation products and which was formed slowly "in vivo". HEINEKAMP (1922) found that oxidized morphine produced the phenomenon in the mouse in a shorter time than did larger doses of untreated morphine.

v. LEERSUM (1918) maintained that mechanical or chemical stimulation of the rectum in mice produced an exact imitation of the Straub-Hermann reaction to morphine. He concluded that this reaction was the result of vesical and anal spasm of spinal origin.

MACHT (1919)(1920) regarded the Straub-Hermann phenomenon as being due, at least in part, to a peripheral effect of morphine. He showed that in respect to their action on plain muscle, the opium alkaloids fell into 2 groups; the piperidine-phenanthrene group of which morphine is the principal member and the benzyl-isoquinoline group of which papaverine is the principal member. Experiments with piperidine hydrochloride revealed at once that piperidine was a powerful stimulant of smooth muscle causing an increase in its tonicity. He further revealed that sodium phenanthrene sulfonate had very little effect on the contractions and tonicity of isolated smooth muscle organs. The biological phenomenon was therefore ascribed to the peripheral effect of the piperidine portion of the morphine molecule and Macht did find that when a suitable dose of piperadine hydrochloride was injected into a mouse or rat, a condition resembling the Straub-Hermann effect was produced soon after the injection.

HEINEKAMP (1922) disagreed with the theories which did not consider the phenomenon as a direct spinal cord stimulation. He

considered the Straub-Hermann phenomenon the result of spinal cord stimulation and not specific for opium alkaloids, since the same results were produced by other cord stimulants as well as by morphine and since the phenomenon occurred after the removal of the rectum and bladder.

F. Efficiency of Methods

The efficiency of the methods devised for the recovery of morphine was tested by control experiments. To check the efficiency and applicability of the method, each investigator added known quantities of morphine to tissue, blood, urine or feces. With progressive improvements in the methods, isolation of smaller amounts of alkaloid was attempted.

1. PERCENTAGE RECOVERIES

a. Blood

Table 1.

Investigator	Morphine added (a) mg.	Blood Vol.(b) ml.	Ratio (a:b)	Recovery %
TAUBER (1890)	75.	100	1:1300	93.3
WACHTEL(1921)	50.	40	1:800	99.0
TERUUCHI & KAI (1927)	50.	100	1:2000	90.0-92.0
BALLS & WOLFF (1928)	8.3	25	1:3000	84.0
	35.2	25	1:830	101.0
IKESHIMA (1933)	4.5	5	1:1100	94.0-100.0
		Average	1:1150	
			85 mg. per 100 ml. blood	

b. Urine

Table 2.

Investigator	Morphine added (a) mg.	Urine Vol.(b) ml.	Ratio (a:b)	Recovery %
CLOETTA (1905)	50.0	1500	1:30,000	96.0
OSHIKA (1919)	5.0	200	1:40,000	0.0
	15.0	200	1:13,300	50.0-76.0
WACHTEL (1921)	40.0	500	1:12,500	107.0
FRY, LIGHT, TORRANCE & WOLFF (1929)	5.0	100	1:20,000	92.0-98.0
TO & RI (1938)	10.0	1000	1:100,000	91.0-93.0
	20.0	1000	1:50,000	95.0-98.0
OBERST (1939)	2.0	70	1:35,000	91.0
	0.05*	50	1:1,000,000	150.0
		Average	1:22,000	
			4 mg. per 100 ml. urine	

*Not included in the average

Table 3.

Investigator	Morphine added (a) mg.	Urine Vol.(b) ml.	Ratio (a:b)	Recovery %
OSHIKA (1919)	24.0	200	1:8000	48.0-64.0
	65.0	200	1:3000	64.0-72.0
FRY, LIGHT, TORRANCE & WOLFF (1929)	33.0	100	1:3000	97.0-100.0
		Average	1:4000	
			25 mg. per 100 ml. urine	

c. Tissue

Table 4.

Investigator	Morphine added (a) mg.	Tissue Wt.(b) gm.	Ratio (a:b)	Recovery %
BABEL (1904)	750	7.0	1:7	97.8
RUBSAMEN (1908)	46	4.0	1:90	105.0
	94	4.0	1:40	100.0
	46	1.2	1:30	97.8
	46	1.0	1:30	97.4
		Average	1:40	
			2500 mg. per 100 gm. tissue	

Table 5.

Investigator	Morphine added(a) mg.	Tissue Wt.(b) gm.	Ratio (a:b)	Recovery %
RÜBSAMEN (1906)	50.	20	1:400	91.6
GOTTLIEB & STEPPUHN	57.8	—	-----	91.0-98.0
(1910)	51.3	—	-----	96.0
TERUUCHI & KAI (1927)	68.4	—	-----	89.0-94.0
	50.0	12	1:240	92.0
BALLS & WOLFF (1926)	50.0	10	1:200	91.0
	17.0	13	1:800	100.0
	75.7	60	1:800	73.0-80.0
	30.3	20	1:700	79.0-81.0
		Average	1:400	
		250 mg. per 100 gm. tissue		

Table 6.

Investigator	Morphine added (a) mg.	Tissue Wt.(b) gm.	Ratio (a:b)	Recovery %
WACHTEL (1921)	100.0	120	1:1200	107.
	50.0	100	1:2000	104.
BALLS (1926)	2.6	12	1:4000	77.
	10.5	24	1:2300	75.
	9.3	21	1:2300	71.
HOTTA (1932)	20.0	53	1:2600	80.
	20.0	60	1:3000	68.
	10.0	53	1:5300	79.
	10.0	67	1:6700	75.
IKESHIMA (1933)	5.0	—	-----	93.-96.
PLANT & PIERCE (1933)	3.0	20	1:7000	95.-97.
		Average	1:3000	
		25 mg. per 100 gm. tissue		

Table 7.

Investigator	Morphine added (a) mg.	Tissue Wt.(b) gm.	Ratio (a:b)	Recovery %
BALLS (1926)	1.7	25	1:14,000	112.
	2.5	26	1:10,000	50.
HOTTA (1932)	1.0	62	1:62,000	66.
	1.0	68	1:68,000	52.
PLANT & PIERCE (1933)	0.9	20	1:20,000	102.
	0.3	20	1:70,000	110.
	1.0	20	1:20,000	99.
	0.6	40	1:70,000	102.
DAUBNEY & NICKOLLS (1937)	37.0	400	1:10,000	84
		Average	1:23,000	
		4.5 mg. per 100 gm. tissue		

d. Feces

BALLS and WOLFF (1928) added 30 mg. morphine to 40 gm. of dried feces (1:1300) and recovered 89 to 98 per cent. PIERCE and PLANT (1932) added from 2 to 20 mg. morphine to 10 gm. dried feces (1:5000-1:500) and recovered from 97 to 100 per cent. ELLINGER and SEEGER (1934) recovered 90 per cent. of 12 mg. morphine added to feces. On the other hand FRY, LIGHT, TORRANCE and WOLFF (1929) stated that the results obtained on control feces determinations were variable and unsatisfactory.

2. EQUATION OF DATA

By tabulating the data from all these control experiments and then equating the amounts of material sampled, i.e. per 100 parts, the differences in the average amounts of morphine isolated from each material becomes evident. In the isolation of morphine from urine, 2 groups with average amounts of 4 mg. (1:22,000) and 25 mg. morphine in 100 ml. (1:4000) are discernible. For blood it is 50 mg. morphine in 100 ml. (1:2000). In tissues 4 groups are evident: 2500 mg. (1:40), 250 mg. (1:400), 35 mg. (1:3000) and 4.5 mg. (1:23,000) morphine in 100 gm. In each succeeding group the ratio is approximately 10 times the value of the preceding one. With decreasing quantities of morphine isolated, the greater becomes the variation in the percentage recoveries. For blood with an average of 85 mg. morphine added, the recoveries lie within 84 to 100 per cent. For urine, in which smaller amounts were added than in the blood, the recoveries are more divergent, 0-107 per cent. For the tissues, as the added amounts decrease the range of recoveries becomes more divergent. For the group with

the largest amounts, the recoveries are from 97 to 105 per cent.; in the second group (200 mg.) the recoveries are from 73 to 100 per cent.; and in the third group (27 mg.) the recoveries range from 68 to 107 per cent.: while in the last group (3 mg.) the recoveries diverge still further, 50 to 112 per cent. The tissue recoveries with the smallest added amounts show the greatest number of fictitiously high results. HATCHER and GOLD (1929) claimed that they could detect morphine in 1 million parts of blood (0.01 mg. per 100 ml.) but could not estimate it even approximately in that quantity. Furthermore, while the extraction of morphine from tissues and its quantitative estimations were easy when present in relatively high concentration (1:5000 or 20 mg. per 100 ml.) they could not recover more than 85 to 95 per cent. of it when one part was present in 25,000 parts (4 mg. per 100 gm.) of liver or other tissues. The estimation was unsatisfactory in their opinion because of unknown factors, such as adsorption or destruction, which interfered with the extraction.

With acquired experience and skillful manipulation many of the established methods proved adequate where fairly large amounts of alkaloid were concerned. However, for small amounts which so frequently must be determined in urine and feces, their adequacy has still to be proven. The isolation of some of the alkaloid present in high concentration is a simple matter and any one of a number of methods can be used effectively, but the extraction of a few milligrams of morphine from 20,000 times or more its weight of complex substances like blood and tissues, is a wholly different problem. With regard to toxicological analysis, the analyst is not unduly concerned with the determination of alka-

loids in cases where excessive amounts are consumed and death has followed rapidly. In such incidents the bulk of the base is present in the stomach and urine. Quantitative estimations of the alkaloid by the classical extraction methods on the stomach contents and urine are straightforward. The types of cases which are not only of interest, but also of considerable importance are those in which an approximation of a minimum lethal dose of alkaloid had been ingested and disseminated throughout the body. The alkaloid, which is partly destroyed in the body and partly eliminated in the urine, then has to be isolated from the tissues, where it remains in minute amounts such as 1 mg. or less per 100 gm. of tissue.

3. COMPARISON OF METHODS

The quantitative comparison of the various modifications of the Stas-Otto method with the Stas-Otto method itself would be of great interest in evaluating the degree of improvement achieved. Unfortunately, no such comparison has been reported on a large scale, as each author tended to use only his own particular modification. DAUBNEY and NICKOLLS (1937) report such a comparison of the recovery of alkaloids injected into rats. With the Stas-Otto process they obtained a 40 per cent. recovery of the injected alkaloid. Progressive improvements which they tried, including adsorption of the alkaloid on Fuller's earth, yielded recoveries up to 79 per cent. With their own improved procedure a 98 per cent. recovery was obtained. The morphine isolated by their method was moderately pure in contrast to the very crude product obtained by the simple classical method of extraction where the

extracted alkaloids were contaminated with comparatively large amounts of adventitious matter.

IV. FATE OF MORPHINE IN THE ORGANISM

A. Excretion

Since the first recorded case of morphine poisoning in 1823, the need for an exact method of isolation and detection of alkaloids in animal tissues and excreta has become increasingly urgent. The solution to the problem of the fate of alkaloids in the animal body came a little nearer when the sensitivity of the methods fell within the confines of the small amounts of alkaloid sought. It is essential to know the distribution of the alkaloid in the body, i.e. the storage in the viscera in cases of alkaloidal intoxication if one is to establish the metabolic fate of the alkaloid by chemical investigation. The contradictions concerning the elimination of morphine in the urine and feces as well as its distribution in the body are exceedingly numerous. The difficulty of extracting small amounts of morphine quantitatively probably serves to explain many of these contradictions. From the early literature on the excretion of injected morphine there was even a great difference of opinion as to whether this alkaloid was excreted in the urine or from the alimentary tract.

1. ELIMINATION IN FECES

a. After acute poisoning

From the early well known observations it appeared that morphine injected subcutaneously or intramuscularly into the animal soon passed into the circulating blood, but whether it was excreted in the urine or from the alimentary tract was a moot point.

Prior to the quantitative isolation of morphine from these channels, the early experiments differed as to the essential amounts of morphine eliminated in the urine but agreed that quantitatively measurable amounts were found in the feces. TAUBER (1890) found an abundance of morphine in the feces. In acute cases of poisoning with morphine in dogs, FAUST (1900) recovered 41 per cent. within 10 days from the feces of a 11.3 Kg. dog injected subcutaneously with 1.6 gm. morphine hydrochloride. FRENKEL (1910) experimented with frogs (Rana temporaria) and found large amounts of the injected morphine in the intestinal tract, even as much as 66 per cent. after 8 days. His conclusion was that the excretion occurred only through the alimentary canal and the intestines were the chief organ of excretion. After a single large dose of morphine to dogs TAMURA (1919) recovered 23 to 28 per cent. of the administered dose in the feces and only traces of it in the urine. PIERCE and PLANT (1930) on the other hand found that the urine contained more than the feces and DE CAMELIS (1927) found only small amounts excreted in the feces of dogs after a single injection.

b. After chronic poisoning

In acute cases of morphine poisoning or after single doses, the elimination in the feces was immediate and abundant. In chronic cases or multiple doses the elimination decreased. FAUST (1900) showed that the elimination in the feces of dogs after a month of chronic poisoning was 26.5 per cent. of the injected morphine and only 4.2 per cent. after one month and 10 days. The fecal elimination of a second dog whose elimination was 20

per cent. at 8 days was nil after 42 days. After 7 or 8 days of daily injections of 100 mg. into dogs and rabbits, TAKAYANAGI (1924a)(1924b) found no morphine in the feces on the 5th or the 6th day. DE CAMELIS (1927) noted that although small amounts of morphine were excreted by dogs, with increasing habituation the morphine excretion became less. FRY, LIGHT, TORRANCE and WOLFF (1929) studied the fecal excretion of 3 human addicts given daily doses of 0.97, 1.94 and 3.89 gm. morphine respectively for a 4 day experimental period and found it to be 2.2 to 3.0 per cent., 1.0 to 3.5 per cent. and 0.3 to 0.9 per cent. of the total amount. The percentage of morphine eliminated in the feces analyzed never exceeded one-fourth the urinary excretion. The figures obtained for fecal excretion were considered by them to be hardly more than qualitative indications that a relatively small amount of morphine appeared in the feces. PIERCE and PLANT (1930) (1932)(1937) likewise showed that more morphine was found in the urine than in feces at any level of dosage. In their experiments on morphine habituated dogs, the urinary elimination of morphine showed considerable variations from day to day whereas the fecal elimination was more constant. During the withdrawal period the morphine disappeared more rapidly from urine than from feces. The amount in feces of chronic human addicts,according to OBERST (1942), varied with dosage. The average daily bowel excretion which was less than 1 per cent. of the daily dose, was less than that of the kidneys.

2. ELIMINATION IN URINE

a. After acute poisoning

MARQUIS (1896) cited 19 investigators who detected morphine in urine and 7 who were unable to find it. After the quantitative estimation of morphine was instituted by TAUBER (1890), the early estimations of morphine were only of an approximate qualitative nature and the knowledge obtained was based on the color reactions which were claimed to be sensitive to 0.05 mg. morphine. MARQUIS (1896) and GOLDEWIJN (1910), who also failed to detect morphine in urine, were about the last authors to report negative findings. The latter failed to find it in bovine urine after 12 daily injections of 250 mg. morphine hydrochloride.

Recent investigators reported the percentage recoveries and maximum periods of elimination of morphine. DORLENCOURT (1913) showed that less than 1 hour after an intramuscular injection of 150 mg. morphine hydrochloride into rabbits the alkaloid appeared in the urine. The maximum elimination was attained in 2 to 4 hours and ended, in general, after 72 hours when about 4 per cent. of the total dose had been excreted. NEVES SAMPAIO (1922), experimenting with dogs which had received numerous injections, maintained that the morphine could be detected in the urine one hour after the injection, and when there was an accumulation, the elimination could be followed for 4 days after the injection. DE CAMELIS (1927) and KEESER, OELKERS and RAETZ (1933) likewise found the excretion of morphine in canine and rabbits' urine respectively to be completed in 72 hours. The last-named investigators recovered from 3 to 12 per cent. within that period.

v.KAUFMANN-ASSER (1913) asserted that the excretion was completed 48 hours after injection. Of 200 mg. morphine hydrochloride injected into rabbits he could recover from 3 to 25 per cent. in the urine. TERUUCHI and KAI (1927) were able to recover from urine of rabbits injected with 100 mg. morphine, 9 to 10 per cent. 3 hours after injection and 18 per cent., 16 hours after injection.

b. After chronic poisoning

Whether there were any outstanding differences in the excretion of morphine in the urine of animals on single or multiple doses was a debatable point. WACHTEL (1921) studied the excretion of morphine in urine of dogs and in the summary drawn from his experiments with continued administration of morphine, he stated that the total excretion amounted to about 25 per cent. of the administered dose. TAKAYANAGI (1924a)(1924b) injected dogs and rabbits with 8 successive daily doses of 100 mg. morphine, the amount recovered in the urine on the first day varying between 1.6 to 7.8 per cent. The elimination increased to 9 to 25 per cent. of the injected morphine on the third and fourth days and at the end of 7 days it had disappeared entirely from the urine. FRY, LIGHT, TORRANCE, and WOLFF (1929), working with human subjects, showed that, regardless of the daily amounts administered for 4 days-900,1800 or 3600 mg.- the fraction eliminated in the urine was remarkably constant. The average amounts of morphine excreted in the urine for all 3 doses were 10.7, 9.8 and 8.7 per cent. of that administered. NEVES SAMPAIO (1922) was able to recover more than 12 per cent. of the injected dose in

the urine of dogs habituated to morphine. With repeated injections, DE CAMELIS (1927) found morphine in the urine of dogs 48 hours after injection and did not find any 3 days following the injection. PIERCE and PLANT (1930)(1932)(1937) after a series of experiments, concluded that there was no essential difference in the amounts of morphine excreted by tolerant and non-tolerant dogs during the first 7 to 10 days of morphine administration. They, as well as OBERST (1942) working with human subjects, found that the urinary excretion of morphine was roughly proportional to the dose since progressively larger amounts appeared in the urine as the dose per Kg. body weight was increased. The average per cent. of the daily dose excreted during continued administration of morphine was 12.5. A considerable portion of the first dose was retained longer than 24 hours to be excreted later, and consequently the amount of alkaloid excreted usually increased during the first 3 or 4 days of administration. Diuresis was found to be an important factor in the urinary excretion of morphine; in consequence the larger the volume of urine the greater was the amount of morphine excreted. FRY, LIGHT, TORRANCE, and WOLFF (1929) maintained that with human subjects, the length of addiction, quantities taken prior to admittance to hospital, age, body weight, and volume of urine did not influence the rate of excretion. Abrupt withdrawal of morphine in their experiments resulted in a rapid fall in urinary excretion during the first 4 hours, followed by excretion at a much lower and constant level for the next 26 hours. In contrast, whether due to another type of subject used or some other variable, TAKAYANAGI (1924a)(1924b) found that in animals which had received morphine previously, the

morphine was eliminated faster. The maximum elimination was reached in 2 to 4 days.

The mechanism of the decreased morphine excretion is little understood despite many attempts to clarify it. The early contradictory results were explained by the differences in the methods used for the detection of the morphine in the urine.

B. Destruction

1. PARTIAL DESTRUCTION

It seemed unlikely that a substance as readily oxidizable as morphine should remain unchanged in the organism. LANDSBERG (1880) postulated that morphine injected into the animal was destroyed either by a ferment or as the consequence of alkalinity of the blood or by the gases of the blood, so that only the decomposition products were eliminated in the urine and, therefore, only those traces of morphine escaping destruction, were detectable in the urine. ELIASSOW (1882), LAMAL (1888) and MARMÉ (1885) found that in protracted poisoning cases the morphine was eliminated in the urine partly unchanged and partly in the oxidized form (pseudomorphine). In consideration of the similar chemical reaction of morphine and pseudomorphine, DONATH (1886) thought it probable that in those cases where morphine was not found, pseudomorphine was present but was overlooked. On the basis of his own research he concluded that the morphine completely disappeared in the organism and was converted to no other alkaloid which coulld be detected with alkaloidal reagents, but was oxidized to the end products of oxidation. Later investigators, TAUBER (1890), MAR-

QUIS (1896), FAUST (1900), CLOETTA (1903) and GÉRARD, DELÉARDE and RICQUET (1905) attributed partial loss of morphine to oxidation, and considered the oxidation process in the organism undeniable. CLOETTA (1903) postulated that the morphine not held in firm combination with the lipoids of the brain was destroyed elsewhere in the body. A ferment was excluded. In addition to oxidation, GÉRARD, DELÉARDE and RICQUET (1905) assumed that there existed another process. DORLENCOURT (1913) claimed that the elimination of pseudomorphine was extremely small and it was not possible in each case to detect the quantities of this alkaloid. WOLFF, RIEGEL and FRY (1933) determined, from the average, that the normal dog at any dosage level between 2 and 200 mg. morphine per Kg. body weight destroyed 80 per cent. of the total.

2. INCREASED DESTRUCTION IN HABITUATION

The decrease in the morphine elimination in chronic cases led FAUST (1900) to the conclusion, on the basis of these experiments, that the chief factor in morphine tolerance was an increased ability of the organism to destroy the drug. TERUUCHI and KAI (1927) assumed that morphinism was due to the acquired power of the organism to destroy morphine and to excrete it. TAKAYANAGI (1924a)(1924b) explained the phenomenon by an apparent increased velocity of destruction of the morphine in the body of the habituated animal. The results of FRY, LIGHT, TORRANCE and WOLFF (1929) showed clearly that in human addicts the amount of morphine destroyed was proportional to the amount absorbed. At any level of single or repeated dosage between 2 and 200 mg. per Kg., WOLFF, RIEGEL and FRY (1933) found that the normal dog ex-

creted about 20 per cent. of the morphine administered. Tolerant dogs on the same level excreted 17 per cent. In both types of dogs approximately two-thirds of the excreted morphine was recoverable from the urine and one-third from the feces. PIERCE and PLANT (1933) like the preceding experimenters, could find no essential difference in the amounts of morphine excreted by tolerant and non-tolerant dogs during the first 7 to 10 days of administration. They found the urinary excretion of morphine roughly proportional to the dose, progressively larger amounts appearing in the urine as the dose per given body weight was increased. They felt that their experimental work furnished no support for the Faust view that tolerance for morphine was dependent on an increased ability of the organism to destroy it. Such differences as did occur in excretion in the tolerant and non-tolerant dogs were too slight to serve in any way as a basis for such a theory.

C. Storage

Retention of morphine within the animal body as an attendant phenomenon to oxidation was a natural conclusion when the fate of the administered drug was determined by a comparative study of the amount administered and the total amount which was excreted. CLOETTA (1903) postulated that the morphine was taken up by the lipoids of the brain and formed a firm combination which withstood destruction. The absence of morphine in the fecal excretions of his experimental dogs induced TAKAYANAGI (1924a)(1924b) to assume a probably longer retention of the feces in the intestinal tract and, as a consequence, a reabsorption of the elimi-

nated morphine followed by a destruction in the body. TERUUCHI and KAI (1927) stated that habituation was due, in addition to an acquired power of the living organism to destroy morphine, to an augmented power by the muscles to store it in large amounts. Of 100 mg. morphine subcutaneously injected into rabbits, they isolated approximately 34 per cent. of the total from the muscles 3 hours after the injection and 22 per cent. after 16 hours. The observations by PIERCE and PLANT (1932) indicated a storage of unchanged morphine in the tissues during continued administration. Apparently during the early period of administration of a daily dose, a level of saturation and excretion was established which was maintained as long as that particular dose was administered.

D. Conjugation

With regard to the phenol hydroxyl group within the morphine molecule, the possibility of the excretion of morphine as morphine alkyl sulfate was not overlooked. ELIASSOW (1882), after giving very large doses of morphine, could verify a very slight increase of bound sulfuric acid in the urine. He did not succeed in establishing the identity of a morphine sulfuric acid. STOLNIKOW (1883) thought of the possibility of the excretion of a morphine alkyl sulfate. After administering morphine to dogs, he found very little morphine excreted in the urine. The experiments also showed that no essential amounts of morphine went into the urine as "morphine ether sulfuric acid." He also fed synthetic "morphine ether sulfuric acid" to dogs and was unable to detect any of it in the urine. The sulfates in the urine were

found to be unmistakably increased. MARQUIS (1896) stated that morphine was excreted by animals in 3 forms; a free, a "paired" and an "altered" form. This was only an arbitrary subdivision as quantitative methods for morphine were not particularly satisfactory for such determinations. Along with the oxidation of morphine, GÉRARD, DELÉARDE and RICQUET (1905) thought it probable that the organism was capable of transforming the morphine to an unstable sulfonic acid derivative or to an analagous ether, which would decompose with hydrochloric acid. They found notable amounts of morphine and pseudomorphine, after hydrolysis, in the liver, kidneys, spleen and urine. DE CAMELIS (1927) found that the normal reaction of the urine in the course of morphine poisoning was always definitely alkaline. The indican excretion increased and reducing substances, which he apparently considered to be morphine glucuronic acid, appeared abundant. When urine from morphinized animals was boiled for 2 hours with N/40 sulfuric acid, ENDŌ (1938) found a larger quantity of morphine in this urine than in non-acid treated urine. He, like some investigators preceding him, believed that morphine was combined with glucuronic acid. His evidence to support this view was based on the corresponding increased glucuronic acid content of the urine of morphine treated rabbits; it reached its maximum value within 3 hours. The detection of the conjugated morphine has shown that morphine underwent far less destruction in the animal body than was formerly supposed. GROSS, PLANT and THOMPSON (1938) reported, that following injury to the liver by the administration of chloroform, the excretion of morphine in the urine was increased in both tolerant and non-tolerant dogs. The degree of increase in

excretion was more marked in the latter. At the time of this investigation the significance of conjugated morphine was presumably not fully recognized. GROSS and THOMPSON (1940)(1940a), by use of suitable hydrolysis methods, demonstrated that dogs excreted morphine in 2 forms, a "free" and a "combined" form. Non-tolerant animals destroyed very little (10-20 per cent.) of the administered dose, the greater portion of the dose being excreted in a "combined" form which GROSS and THOMPSON postulated as the first step in the detoxification of the alkaloid. The tolerant dogs, on the other hand, excreted only about 50 per cent. of a given dose of which 30 per cent. was found in the "combined" form. The same authors considered it probable that the tolerant animal was capable of destroying a much larger part of the ingested alkaloid. At about the same period OBERST (1940)(1941)(1941a)(1942) also demonstrated a "bound" form of morphine excreted by human addicts, which varied in amounts from 3 to 36 times that of the free form, both being greater with the higher dosage. He was unable to determine with which substance the morphine was conjugated, but conjectured that since it contained the phenolic group and a secondary alcoholic hydroxyl group, it was likely that the drug was excreted largely as a conjugate of glucuronic acid or its lactone form, glucurone. When both the hydroxyl groups of morphine were methylated the conjugating mechanism was lost. THOMPSON and GROSS (1941), studying the combined morphine form excreted in canine urine after morphine administration, further found that there were distinct differences in the amounts of the "combined" morphine in the tolerant and non-tolerant animals. The latter excreted twice as much of the "combined" forms as the

former. The "combined" morphine was excreted in 2 forms: a fraction which was "easily hydrolyzable" by 2 hours' boiling at pH 1 to 3 and the other, the fraction "difficultly hydrolyzable" by autoclaving under 15 pounds pressure with 5 per cent. (by volume) hydrochloric acid. The free morphine fraction was found in the urine of tolerant dogs after excretion of the other fraction had ceased. In the non-tolerant dogs the fraction "difficultly hydrolyzable" of the combined morphine was found to be relatively higher toward the end of the excretion period, the free morphine was low and the fraction "easily hydrolyzable" completely disappeared. The earlier observation that liver damage from chloroform poisoning produced an increase in the free morphine was later confirmed by GROSS (1942) who proved that the "easily hydrolyzable" occurred at some other site. In his experiments the total recoverable morphine was not materially altered and the portion "difficultly hydrolyzable" remained fairly constant; the compound "easily hydrolyzable" appeared as free morphine. Attempts to isolate the bound morphine from urine or to synthesize morphine glucuronide, so far, have met with failure. OBERST and GROSS (1944) made some urinary excretion studies for free and bound morphine in tolerant and non-tolerant dogs, addicts and non-addicts, after administration of morphine sulfuric ether. They could not ascertain whether the bound morphine in the urine was the unchanged substance or whether it was the form in which morphine was usually excreted; i.e. possibly the glucuronide.

Up to this point three factors were considered as depicting the means by which the organism disposed of morphine. The first was the almost immediate excretion of the unchanged alkaloid,

which accounted, on the average, for only a small fraction of the ingested dose. The second was its destruction as measured by the discrepancy between the amount ingested and the amount recovered from the excreta. The third was its elimination in a conjugated form, the recent rediscovery of which attested to a much smaller degree of destruction of morphine than was formerly supposed. Another possibility, still to be discussed, is the absorption or storage of the alkaloid in the tissues from which it is slowly released and excreted.

E. Distribution in Tissues, Body Fluids and Secretions

Most of the present knowledge regarding the morphine content of various biological materials has been derived from analysis of morphinized laboratory animals who usually received considerably more morphine per unit of body weight than human addicts.

1. BLOOD

a. In non-habituation

The value of these studies, even more than the others, depended primarily on the efficiency of the methods employed for the recovery of the alkaloid. The general opinion is that morphine leaves the circulation rapidly. CLOETTA (1903) stated that the morphine completely disappeared from the blood in 20 minutes whereas WACHTEL (1921) could detect none in rabbits' blood 5 minutes after intravenous injection. HATCHER and GOLD (1929) fixed the time interval as 5 to 10 minutes following intravenous injection; only traces of morphine were found in blood of cats after

that time. They found that it did not leave the blood of dogs so rapidly, as small amounts were present after 30 minutes. FLEISCHMANN (1931) noted that the morphine in blood generally disappeared in the course of hours. The maximum concentration was reached mostly within the first hour depending upon the concentration of morphine in the injected dose. In 100 ml. blood of a normal dog the morphine concentration was so small that IKESHIMA (1934) was unable to determine it 1 hour after the subcutaneous injection of 10 mg. morphine hydrochloride per Kg. body weight. He could detect 1.7 to 2 mg. morphine 1 hour after the subcutaneous injection of 100 mg. per Kg. body weight. The work of KEESER, OELKERS and RAETZ (1933) verified the Fleischmann data. In their work, the blood of the guinea pig reached its maximum concentration 30 minutes after subcutaneous injection and remained unchanged for 2 hours. After 24 hours no morphine was present in the blood. TERUUCHI and KAI (1927) injected rabbits subcutaneously with 100 mg. morphine and, 3 to 16 hours later, found a constant amount, 3.2 per cent. of amount injected, in the blood. PLANT and PIERCE (1933) were also unable to detect morphine in blood of dogs 24 hours after injection.

b. In habituation

HATCHER and GOLD (1929) found it impossible to determine any essential difference in the rate of disappearance of morphine from blood of normal and habituated animals. On the other hand, PLANT and PIERCE (1933)(1933a) noted that there was a difference in the manner in which morphine was metabolised by tolerant and non-tolerant dogs. The blood of the former contained more

than that of the latter, 4 to 24 hours later. The results obtained by IKESHIMA (1934), after subcutaneous single or multiple injections of 100 mg. per Kg. body weight of dogs, agreed with these and showed that the morphine was retained in the blood longer in the habituated than in the normal animals. OBERST (1942) could offer no explanation for the curious fact that no morphine, free or bound, was found in the blood of human addicts except that the morphine may have been present in concentrations less than 0.1 mg. per 100 ml. blood, the limit of sensitivity of his method.

c. Destruction

LAMAL'S (1888) failure to detect morphine in blood led him to the assumption that the morphine was transformed to "oxymorphine" (pseudomorphine) in the circulating blood. CLOETTA (1903) claimed that morphine was not destroyed in the blood in any noteworthy amounts. From "in vitro" experiments, TERUUCHI and KAI (1927) could detect no destruction of morphine added to blood. FLEISCHMANN (1931) concluded that the decrease in the morphine concentration in the blood must be explained by a storage in the organs rather than by a destruction in the blood. When a small amount of morphine was present in blood of human addicts OBERST (1942) considered the possibility that the morphine could escape detection by adherence to the protein, later precipitated, or by its resistance to solution when the sample was prepared for solution.

d. Red cell-plasma distribution

TERUUCHI and KAI (1927) reported almost equal amounts of morphine in the red cells and plasma, 48 to 54 per cent. in the red cells and 39 to 40 per cent. in plasma, of 50 mg. added to blood "in vitro". MULL (1936) confirmed the findings of Teruuchi and Kai. FLEISCHMANN (1931) claimed that when morphine was added to blood "in vitro", the ratio of the ultimate morphine concentration in the blood cells to that in the serum was between 1.7 to 2.5. He pointed out a fact, overlooked by the previous investigators, that the ratio of the morphine concentrations was influenced by variations of the hematocrit, the type of animals used and the method of morphine addiction "in vitro" or through injection. The time of equilibration for the blood was not given by any of the investigators.

2. TISSUES

a. Liver

MARQUIS (1896) found that one-half hour after subcutaneous injection 38 per cent. of the morphine was deposited unchanged in a cat's liver, the amount gradually dwindling. In frogs (Rana temporaria) FRENKEL (1910) demonstrated that a part of the morphine was deposited in the liver where it reached its maximum concentration within 4 hours after subcutaneous injection of 30 mg. morphine hydrochloride. He considered this organ as the main depot for morphine storage. Of 100 mg. morphine injected subcutaneously into rabbits, TERUUCHI and KAI (1927) recovered 4.4 per cent. of the total in the liver 3 hours after in-

jection and 10.8 per cent. after 16 hours. With an overwhelming dose, 11.3 mg. morphine hydrochloride (1.1 gm/Kg) intravenously injected into a dog over a 5 hour period, WOLFF, RIEGEL and FRY (1933) could recover only 0.5 per cent. in the liver 35 minutes after the injection ended. In a dog's liver 4 hours after subcutaneous injection of 50 mg. per Kg., PLANT and PIERCE (1933) isolated 0.8 per cent. of the total injected after 4 hours and 0.3 per cent. after 24 hours. KEESER, OELKERS and RAETZ (1933) found the maximum concentration of 45 mg. per 100 gm. liver of guinea pigs from 15 minutes to 2 hours after subcutaneous injection of 0.4 gm. morphine hydrochloride per Kg. body wiight, i.e. about 11 per cent. of the dose was found in the liver. At 24 hours none was found in this organ.

Experiments on chronic poisoning with morphine offered information as to the extent the daily intake of morphine affected the increase of the alkaloid in the body. With daily doses up to 0.3 gm. morphine for over a month, FAUST (1900) could detect none of the alkaloid in the liver. KEESER, OELKERS and RAETZ (1933), on the contrary, were able to recover from 9 to 18 mg. per 100 gm. from the liver of guinea pigs subcutaneously injected daily with 100-150 mg. morphine hydrochloride for a period of 3 to 6 months.

b. Muscle

Muscles were considered by FRENKEL (1910) to be a depot for morphine storage, second to liver. In frogs' muscle, 5 hours after subcutaneous injection, he obtained 50 mg. per 100 gm. and 24 hours after, 37 mg. morphine per 100 gm. tissue. The

results of KEESER, OELKERS and RAETZ (1933) on a guinea pig injected subcutaneously with 400 mg. morphine hydrochloride per Kg. body weight, agreed with those of Frenkel. The muscles, which yielded their maximum amount (24 mg. per 100 gm.) within the first hour, stored less morphine and released it sooner than the liver. HATCHER and GOLD (1929) stated that after leaving the circulation, morphine was stored largely in the skeletal muscles and in the kidneys. This view was also held by TERUUCHI and KAI (1927) who maintained that in cases of acute intoxication the muscles were the most important tissues in which morphine was easily held. Three hours after subcutaneous injection of 100 mg. morphine into rabbits they located 34 per cent. of the dose in the muscles and 21.6 per cent. after 16 hours. After 32 days of daily subcutaneous injections with amounts varying from 40 to 340 mg. (total-4.7 gm.), morphine into rabbits they still were able to recover 3.6 per cent. in the muscle. Of the individual tissues, PLANT and PIERCE (1933)(1933a) found that the muscle yielded the largest amount of morphine but the concentration in this tissue was of the same order as the other tissues. WOLFF, RIEGEL and FRY (1933) also showed that a considerable portion of the injected morphine was taken up by the muscles. Of 11.3 gm. morphine hydrochloride (1.1 gm./Kg.) they intravenously injected into a dog over a 4 hour period, 21 per cent. of the dose was located in the muscles, 35 minutes after the cessation or complete injection.

c. Brain

Neither FRENKEL (1910) nor WACHTEL (1921) could show the presence of morphine in the brains of frogs or rabbits respectively,

at any time. HATCHER and GOLD (1929) and FLEISCHMANN (1931) found only traces of morphine in the brain. After the subcutaneous injection of 86 mg. morphine per 100 gm. body weight into a guinea pig, Fleischmann recovered only 0.15 mg. morphine from the entire brain (323 gm.) 30 minutes later. KEESER, OELKERS and RAETZ (1933) found a constant amount (4.5 mg. per 100 gm.) from 15 minutes to 8 hours after injecting guinea pigs with 0.4 gm. morphine hydrochloride per Kg. body weight. The morphine content of the brain was nil after 16 hours. With an excessive dose of 11.3 gm. morphine hydrochloride (1.1 gm. per Kg.) intravenously injected into a dog over a 5 hour period, WOLFF, RIEGEL and FRY (1933) located as little as 0.15 per cent. of the dose in the brain 35 minutes after the completion of injection. KEESER and KEESER (1928) claimed that morphine in the brain was found chiefly in the corpus striatum and thalamus region but could not be demonstrated under the same conditions in the pons, medulla oblongata and cerebellum. IKESHIMA's (1934) results indicated that with the injection of a relatively small amount (10 mg.) morphine hydrochloride per Kg. into dogs as compared with the injection of a relatively large amount (100 mg.) morphine hydrochloride per Kg., only about twice the smaller amount (2.1 compared to 4.5 mg. per 100 gm.) was deposited in the brain within 1 hour and not a proportionately larger amount (10 times) as one would expect.

In chronic poisoning of dogs with daily doses worked up to 0.3 gm., FAUST (1900) asserted that the brain contained no morphine. FLEISCHMANN (1931), on the contrary, found a trace (0.1 mg.) in the entire brain of guinea pigs after 3 weeks' treatment with 80 mg. morphine hydrochloride thrice weekly. IKESHIMA (1934)

claimed that the greater the habituation, the smaller was the amount of morphine found in the brain tissue.

d. Kidney

According to HATCHER and GOLD (1929) the kidney was one of the tissues in which morphine was stored abundantly after leaving the circulation. As much as 81 mg. morphine per 100 gm. tissue was taken up by the kidneys of a dog in WOLFF, RIEGEL and FRY's (1933) experiment in which an exorbitant dose of 11.3 gm. morphine hydrochloride (1.1 gm. per Kg.) was intravenously injected over a 5 hour period and the morphine in the tissue determined 35 minutes after completion of the injection. KEESER, OELKERS and RAETZ (1933) recovered from the kidneys of guinea pigs subcutaneously injected with 400 mg. morphine hydrochloride per Kg. body weight, 95 mg. morphine per 100 gm. tissue 4 hours after injection and 32.5 mg. per 100 gm. at 24 hours. FAUST (1900) could not detect morphine in the kidneys of a dog subcutaneously injected daily for 42 days.

e. Intestines

TERUUCHI and KAI (1927) found in the intestinal wall approximately 12 per cent. of 100 mg. morphine injected subcutaneously into rabbits 16 hours previously. None was found in the intestines 3 hours after morphine ingestion. WOLFF, RIEGEL and FRY (1933) recovered 1 per cent. of the morphine from the intestinal tract 35 minutes after the intravenous injection into a dog of 11.3 gm. morphine hydrochloride over a 5 hour period.

f. Lungs

PLANT and PIERCE (1933) recovered from the lungs of dogs about 1.5 mg. morphine of a total of about 450 mg., 4 hours after subcutaneous injection and about 0.38 mg. 24 hours after. The results were identical for tolerant and non-tolerant animals.

g. Bones

WOLFF, RIEGEL and FRY (1933) located in the bones 5.5 per cent. of 11.3 gm. morphine hydrochloride shortly after it was intravenously injected into a dog. The injection was spread over a period of 5 hours.

h. Placenta

The work of SHUTE and DAVIS (1933) indicated that the placenta did not retain morphine and was not an important barrier to the passage of morphine from the mother to the fetus.

3. SECRETIONS

a. Stomach

A trace of morphine has been found present in the gastric contents after subcutaneous or intravenous injections. BONGERS (1894) tested for morphine in the gastric contents at 14 and 45 minutes after subcutaneous injection of 100 mg. morphine hydrochloride into dogs and found positive results at the 45 minute level. FRENKEL (1910) claimed to have detected a trace of morphine in the frog's stomach. HATCHER and DAVIS (1926) also, found only traces of morphine excreted into the stomach of the

cat and dog after the subcutaneous, intramuscular or intravenous injections of amounts varying from 56 to 982 mg. morphine. OBERST (1942) found both free and bound forms of morphine in the gastric contents of human addicts.

b. Saliva

ROSENTHAL (1893) appeared to have been the first to demonstrate that morphine was eliminated in the human saliva. Chemical methods of detection were used to determine between 0.05 to 0.2 mg. morphine which saliva was estimated to contain. The saliva tests were negative on the first and second days after daily administration and then positive for 1 or 2 days after the dosage was discontinued. MUNCH (1934a) used the biological method for the estimation of morphine in equine saliva. Of 0.22 to 2.2 mg. morphine per Kg. injected into 9 horses, 15 minutes later 4 showed negative and 5 positive results. After 30 minutes the saliva of all but 1 horse showed a positive test for morphine. OBERST (1942) failed to find morphine, either free or bound, in human saliva.

c. Bile

Only traces of morphine, according to HATCHER and GOLD (1929), were excreted in the bile in cats and dogs. KEESER, OELKERS and RAETZ (1933) found the morphine content of the gall bladder contents essentially higher than that of the blood in dogs with habituation periods from 17 to 40 days. OBERST (1942) found only the bound form of morphine in the bile of human addicts to the extent of 0.07 mg. per 100 ml.

d. Perspiration

OBERST (1942) claimed to have shown for the first time that morphine was excreted in the perspiration.

e. Milk

KOLDEWIJN (1910) failed to detect morphine in bovine milk after the daily injection of 250 mg. morphine hydrochloride for 12 days. TERWILLIGER and HATCHER (1934) stated that a specimen of milk drawn from a normal woman about 7 hours after the administration of 16 mg. morphine sulfate may have contained a trace of morphine. On the other hand, the milk from a woman, addicted to morphine, after giving birth showed no trace of morphine.

F. Destruction of Morphine in Habituation

Diametrically opposing views have developed concerning the behavior of the morphine habituated body toward morphine. Some authors claimed that the body possessed certain strong capacities to destroy morphine and that these capacities were not decreased by habituation. FAUST (1900) came to the conclusion that the chief factor in the acquired morphine tolerance was an increased ability of the organism to destroy morphine. CLOETTA (1903) made the claim that, in habituation, the power of the lipoids of the brain to combine with morphine increased, but at the same time an increase in the rate of decomposition of the alkaloid also took place. In studying the difference in the rate of disappearance of morphine in rats of 7 and 14 days' habituation, TAKAYANAGI (1924a)(1924b) noted that the velocity of destruction was appar-

ently increased. He, forthwith, postulated that the habituation to morphine consisted, in addition to an increased destruction in the animal body, of an increased immunity of the cells toward morphine. DORLENCOURT (1913a) agreed that the destruction of morphine was greater in the most habituated dogs. The non-habituated dogs destroyed 15 per cent. of the added morphine while the habituated ones destroyed from 31 to 45 per cent. when the dogs were sacrificed 12 hours after the last injection. TERUUCHI and KAI (1927) were able to recover in acute intoxication in rabbits up to 90 per cent., but in chronic morphinism of about 32 days only 16.4 per cent. of the total amount of injected morphine. They also concluded that the morphinism was due to the acquired power of the living organism to destroy morphine, besides an augmented power to store large amounts in the muscles.

On the other hand, HATCHER and GOLD (1929) found no evidence that the tissues of the habituated dog acquired an essentially greater capacity for destroying morphine, except insofar as habituated animals tolerated large doses with less disturbances of circulation and presented larger amounts of morphine for the tissues to destroy. They found no essential difference in the average amounts of morphine present in the tissues of tolerant and in those of non-tolerant dogs after approximately similar intervals of time. That there was a difference in the manner in which morphine was handled by tolerant and non-tolerant dogs was not denied by PLANT and PIERCE (1933)(1933a). On the basis of their excretion experiments they found no support for the Faust view that increased destruction was an important factor in tolerance . Their own results seemed to indicate a storage of morphine in tol-

erant dogs, in a form that was not readily extracted by the usual methods. IKESHIMA (1934) asserted that the greater the habituation the smaller was the amount of morphine found in the brain tissue. He, therefore, assumed that the binding ability of the brain tissue for the alkaloid was strongly diminished by habituation. The experimental work of HINOHARA (1937) indicated that, "in vitro", blood and muscles of tolerant or non-tolerant animals had no capacity to metabolise morphine at room temperature or at 39°C. KUWAHARA (1937), likewise, pointed out that the ability of the liver of non-tolerant rabbits to destroy morphine "in vitro" was not increased by habituation to the drug.

1. SITE OF DESTRUCTION

The general agreement that the normal body possessed a considerable ability to destroy morphine quite naturally led to the investigation of the seat of this transformation. Several organs were proved capable of destroying the alkaloid.

a.Liver

TAUBER (1890), on the basis of perfusion experiments, concluded that the liver had the capacity to destroy morphine. FABRE (1924), IWASE (1932) and KO (1937), repeating the perfusion experiments, concurred with Tauber's view. To Ko it appeared that this ability was increased in proportion to the period of addiction until a toleration level was reached by the habituated animal. DORLENCOURT (1913a) maintained that the liver's ability to destroy morphine increased at the same rate as that at which tolerance was established and was proportional to it. KUWAHARA (1937) found

that the capacity of the liver tissue of non-tolerant rabbits to destroy morphine "in vitro" was not increased by habituation to the alkaloid. While GROSS, PLANT and THOMPSON (1938) pointed out that the liver was one of the tissues that destroyed a considerable portion of the administered morphine, GROSS (1942) later intimated that the morphine suffered far less destruction in the animal body than had formerly been presumed. The conjugation of the "easily hydrolyzable" fraction of morphine occurred in the liver.

b. Kidney

TAUBER (1890) considered the kidney as another tissue capable of destroying morphine. By perfusion of the rabbit's kidney, IWASE (1934) determined that less was retained by the kidney, which had less ability to destroy the morphine than the liver. Although HATCHER and GOLD (1929) found the kidneys fixed morphine abundantly after leaving the circulation, they made no claim that it was destroyed there.

c. Brain

CLOETTA (1903) made a point of the affinity which the lipoids of the brain tissue had for morphine. The part of the alkaloid which was not held by the brain, he propounded, was destroyed elsewhere in the body. RÜBSAMEN (1908) incubated morphine with the brain of a normal animal under a stream of oxygen and failed to find 34 to 44 per cent. of the added morphine. In a similar experiment with the brain of an habituated animal the

added morphine was destroyed to the extent of 71 to 100 per cent. He, therefore, reasoned that an oxidative process of the morphine was taking place in the brain.

d. Muscle

HATCHER and GOLD (1929) recorded that large quantities of morphine were stored in the muscle tissues. Whether it was destroyed there or not was not mentioned. HINOHARA (1937) mentioned that "in vitro" experiments of tolerant and non-tolerant animal muscle tissues showed that they had no capacity to transform morphine at room temperature or 39°C.

e. Placenta

HIGUCHI (1909) reported from his "in vitro" work with human placenta that this tissue was unconcerned in the destruction of morphine in the human organism.

G. Stability of Morphine

1. IN SOLUTION

That morphine is stable in acid solution and unstable in alkaline solution is an accepted fact. Recent work has added further contributions to the general knowledge of its stability, especially of morphine solutions that have been subjected to various heating periods. TAKAYANAGI (1924), in advancing his method of extraction, showed proof that long boiling and drying on sand of a morphine hydrochloride solution was not harmful. ŌSHIKA (1919) determined the amounts of morphine present in a

chloroform extract of urine after boiling for varying periods of time; of 50 mg. morphine added to this extract, the maximum recovery was 89 per cent. after 5 hours' heating; 75 per cent. after 10 hours' heating and 59 per cent. after 20 hours' heating. At the beginning of the heating period the morphine was completely soluble but after several hours a yellow flock appeared which increased with the heating time. This precipitate showed all the reactions and properties of morphine. Similar experiments using amyl alcohol in place of chloroform produced no turbidity. Chloroform extracts of feces and liver did not produce the same changes as the urine chloroform extracts. BALLS and WOLFF (1928) endeavored to show how easily morphine could be decomposed in neutral or alkaline solutions, particularly on evaporation under conditions frequently occurring in the laboratory. To evaluate this error, they placed known amounts of pure morphine in known volumes of liquid and evaporated the solution from dishes. The morphine underwent oxidation, the residue increased in weight, became brown and resinous in appearance. The loss of morphine on evaporation from water, with an evaporation time of 3.5 to 16 hours, was 16 per cent. of the original; from dilute ammonia solution with an evaporation time of 5.5 to 9.5 hours, 21 per cent; from alcohol with an evaporation time of 1.5 to 12 hours, 2.7 per cent; from freshly washed chloroform with an evaporation time of 1 to 6.5 hours, 2.4 per cent. DIETZEL and HUSS (1928) were able to follow the decomposition of morphine with an ultraviolet spectrograph which has a pronounced selective absorption curve whose number and position in the spectrum can be defined with certainty. They heated a morphine hydrochloride solution under reflux at a

constant temperature (98°C.) for varying periods of time. The solution heated for 30 minutes corresponded to one not heated, i.e. no change in the morphine occurred. With 60 and 120 minutes of heating the morphine, there was a definite displacement of the absorption curve in the visible part of the spectrum. A brownish color developed after heating the solution for 120 minutes. In strongly acid (pH 3.2) and weakly acid (pH 6.0) solutions, they found that morphine suffered no change in its chemical structure when heated from 60 to 120 minutes. Morphine solutions at pH 6.0 up to 11.7, even after 30 minutes' heating, showed considerable spectral changes. The absorption spectrum of a morphine solution at 20°C. and at 98°C. at a pH 3.2 into which air streams were injected, were identical to one corresponding without air passing through. Morphine solutions on standing gradually deteriorate. RISING and LYNN (1932) found that aqueous solutions of morphine contained only 4 to 5 per cent. of the original alkaloid at the end of a year.

2. IN PUTRIFIED TISSUE

The destruction of morphine in the tissues has been the subject of controversy, especially when its stability in contact with putrifying biological material for varying periods of time has been considered.

OGIER (1911) stated that he frequently had been unable to detect morphine after exposure in viscera putrified from 2 to 4 weeks. In contrast, WOODMAN and TIDY (1887) claimed to have isolated the alkaloid from the stomach of a body exhumed after 4 months. Subsequent workers agreed that morphine could be de-

tected in the cadaver or putrifying meat but disagreed about the maximum limit of time. GÉRARD, DELÉARDE and RICQUET (1905) were able to detect morphine and a little pseudomorphine in the liver and kidneys of a dog permitted to putrify for 22 days. FABRE (1924) always obtained a positive test for morphine isolated at the end of 2,4,8,15,30 and 45 days after its addition to hashed veal liver left at a temperature of 20° to 25°C. NEGELVOORT (1898) could prove chemically the presence of morphine in a corpse about 2 months after death. After 9 months of decaying process, IPSEN (1913) succeeded in recognizing morphine chemically. DOEPMANN (1915) detected unchanged morphine in putrified meat to which it had been added 11 months previously. AUTENREITH (1901) was of the opinion that morphine was only slightly decomposed even after long periods of putrefaction. After 18 months of continuous putrefaction, he found 200 mg. morphine in what was left of the cadaverous material. The boldest statement on the subject was made by GRUTTERINK and van RIJN (1915) by their claim that they could detect this alkaloid with certainty after it had been in the cadaver for 2 years and 6 months. Regardless of the condition of the tissues or presence of ordinary preservatives, RISING and LYNN (1932) were certain that morphine could be detected in a body about a year after death. Decomposition of the alkaloid commenced immediately and continued, more or less gradually, until the alkaloid was completely destroyed. This process, in their opinion, required a little more than a year. Within a month after death 90 per cent. was capable of detection, 70-80 per cent. after 3 months, and it was doubtful whether 50 per cent. could be recovered from any tissue after decomposition had progressed

longer than 8 months. The rate of decomposition, they asserted, was affected very little by the agents used to prevent putrefaction. If there was any real effect it was an accelerating, rather than a retarding one.

V. PSEUDOMORPHINE

(Oxydimorphine, Oxymorphine, Dehydromorphine)

When morphine undergoes gentle oxidation, some of the substances formed resemble morphine in many chemical properties. The first of these oxidation products is theoretically, pseudomorphine, formed by the removal of 1 equivalent of hydrogen per morphine molecule. Besides pseudomorphine, a large number of other substances are found during the early stages of oxidation. Their behavior with many of the alkaloidal reagents is like that of morphine; they are precipitated by the complex acids of tungsten and molybdenum, nearly as completely as morphine itself. Because of the probable formation of pseudomorphine at the very onset of morphine oxidation its detection and isolation should receive more consideration. Only a few attempts have been made to do this. LAMAL (1888) pointed out that in toxicological research on morphine, both morphine and its first oxidation product, pseudomorphine, should be sought in the blood, urine and vascular organs. The importance of the detection of pseudomorphine is due to the fact that all the morphine could be transformed into pseudomorphine and that its discovery was a new proof for the presence of morphine. GÉRARD, DELÉARDE and RICQUET (1905) advocated the modification of the Stas-Otto procedure in order to do toxicological research on morphine or preferably on its derivatives formed in the organism. BALLS (1926) agreed that pseudomorphine would not be detected as it would be excluded by the various processes of protein precipitation, clarification and alkaloidal extraction which in one form or another invariably accompany the

morphine methods. Because of its insolubility in the organic solvents used in the various methods of extraction, PIERCE and PLANT (1932), also believed that pseudomorphine has not been estimated, along with morphine, in the analysis of urine and feces, although this base gave quantitatively the same diazo color reaction as morphine. An apparently contradictory statement was made by BALLS and WOLFF (1928) when they claimed that such oxidation products, unless intentionally separated, were likely to follow the morphine and be determined as such, thus showing a nearly complete recovery as claimed by some investigators with their control experiments. No distinction was possible by such methods between the oxidized morphine formed during analysis and that pre-existing in the material analyzed. Since more than 1 oxidation product of morphine is involved the statements are not so contradictory on second consideration. The other oxidation products still retain many reactions of morphine and are soluble in most morphine solvents.

A. Production of Pseudomorphine

The presence of pseudomorphine has been reported in a number of cases, such as by mild oxidation by gold and silver salts, by oxygen, potassium permanganate, and hydrogen peroxide in alkaline solutions. It has been produced by oxidation with potassium ferricyanide (GRIMBERT and LECLERE (1914), DIETZEL and HUSS (1928)). With potassium ferricyanide Dietzel and Huss obtained a 65 per cent. yield as compared to one of 25 per cent. using potassium nitrate as the oxidizing agent. It has also originated biochemically by the action of oxidizing enzymes. BOUGAULT (1902) found,

as did BOURQUELOT (1896), that the juice of certain mushrooms (Russula delica) oxidized morphine to pseudomorphine. GONNERMANN (1906) found a similar conversion with a plant tyrosinase. The conversion of morphine by a vegetable enzyme gave rise to the theory that this alkaloid introduced into the organism might similarly show the same transformation under the influence of an enzyme.

B. General Reactions

1. PSEUDOMORPHINE

Pseudomorphine, in the ultraviolet spectrograph work of DIETZEL and HUSS (1928), showed characteristic spectral differences from morphine. The ultraviolet absorption was on the whole strongly displaced with the long wave lengths predominating. It ran essentially linear and did not show the strong marked absorption band characteristic of morphine in the vibration frequency range of 3400 to 3800. This alkaloid is a weaker base than morphine but a stronger acid. As yet, its isoelectric point has not been properly determined but is in the vicinity of pH 8.0. It has a wide isoelectric range. By virtue of this property, its separation from morphine by precipitation in slightly acid solution has been worked out.

The base is soluble in aqueous and alcoholic ammonia solution, more readily in the former. It is not precipitated by excess ammonia. Most acids and caustic alkalies are good solvents for this base. DONATH (1886) found pseudomorphine to be insoluble in water, alcohol, ether and chloroform but readily soluble

in hot amyl alcohol. LAMAL (1888) used ammonia-amyl alcohol as a solvent for morphine and pseudomorphine and BALLS (1926) took advantage of the solubility of both in benzyl alcohol. BOUGAULT (1902) determined its solubility as 5 mg. per 100 ml. each of chloroform, amyl alcohol, ethyl acetate and ether. BALLS (1926a) found only traces dissolved in hot butyl and amyl alcohols. This fact, in the opinion of BALLS (1926a), was of the utmost importance in regard to the usual methods of alkaloidal isolation from biological material. It is doubtful whether these methods would reveal the presence of pseudomorphine.

2. SALTS OF PSEUDOMORPHINE

Pseudomorphine hydrochloride is a white, poorly crystallized substance. Its solubility in water is about 1 part in 125. The aqueous solution hydrolyzes on dilution, slowly precipitating the free base.

The sulfate resembles the hydrochloride except that it is less soluble. In methyl alcohol and ethyl alcohol the hydrochloride is insoluble and the sulfate quantitatively so. This is a characteristic difference from the morphine.

Trichloracetic acid precipitates an insoluble salt which is not readily redissolved by alcohol or acetic acid. This is another distinction from morphine.

Silicotungstic acid throws pseudomorphine out of acid solution as a finely divided, gelatinous, nearly white precipitate which may be coagulated by electrolytes. When dried at 120°C. it differs from the corresponding morphine salt in color and in containing no water of crystallization. It also differs from mor-

phine in being more insoluble in dilute acids and alcohol and in being able to be completely precipitated from hot solution.

While silicotungstates of morphine and other oxidation products, proteoses and peptones are readily soluble in phosphate buffers of pH 6.5, pseudomorphine silicotungstate is insoluble at pH 8.0. This difference gives a method of separating pseudomorphine from these similarly reacting substances.

The isoelectric point of the base is such that the hydrolysis produced in nearly neutral solutions of its salts by such substances as potassium phenolsulfonate or potassium fluoride is sufficient to cause the characteristic precipitation even from fairly dilute solutions. The presence of pseudomorphine in morphine may be recognized by adding such substances to the solution of the mixture or by using an excess of M/5 phosphate buffer of pH 6.5. The morphine remains in solution whereas the pseudomorphine is precipitated immediately.

C. Quantitative Determination

The exceptional insolubility of many salts of pseudomorphine has made several methods of determination possible. Four quantitative methods, 3 gravimetric and 1 colorimetric have been successfully applied.

1. DETERMINATION AS FREE BASE

Pseudomorphine has a wide isoelectric range and since the free base is highly insoluble, BALLS (1926) precipitated it by adjusting the reaction of the solution to pH 7.8. The separated base was filtered on a Gooch crucible, washed with 30 per cent.

alcohol and dried to constant weight. Washing with water could not be used since the base came through the filter in a colloidal condition when all the salts were removed. The solubility of the base is about 2 mg. per 100 ml. water. This method was therefore not recommended for small amounts.

2. DETERMINATION AS SULFATE

Pseudomorphine sulfate is only slightly soluble in strong alcohol and less so in acetone. BALLS (1926) quantitatively precipitated the pseudomorphine from acetone in the presence of sulfuric acid. The precipitate was filtered on a Gooch crucible, washed with acetone and dried at 100°C. Pseudomorphine sulfate x 0.852 = free base.

3. DETERMINATION AS SILICOTUNGSTATE

The precipitation of pseudomorphine silicotungstate is carried out in the same manner as that of morphine silicotungstate. BALLS (1926) found it unnecessary to reduce the volume of the solution before its precipitation, as in morphine, because of the greater insolubility of the pseudomorphine compound. The precipitate was collected on a Gooch filter, washed with acidulated water and then alcohol and dried at 120°C. It contained no water of crystallization. Balls also recommended ignition of the weighed precipitate as a check. The following factors were given:

Pseudomorphine x 0.282 = free base
Ignited oxides x 0.399 = free base
Loss on ignition x 0.970 = free base

DREVON (1935) developed a colorimetric method after the isolation of the pseudomorphine as the silicotungstate. The precipi-

tate dissolved in aceto-sulfuric acid (1-20 by volume of 99 per cent. acetic anhydride and concentrated sulfuric acid) with the formation of a characteristic green color with an absorption band in the red and orange. The maximum color was attained within 30 minutes and was very stable. Water caused the color to disappear. The method was claimed to be specific and 0.1 to 0.3 mg. pseudomorphine in 5 ml. solution could be determined with an error of 5 per cent.

D. Separation of Pseudomorphine from Morphine

The separation of pseudomorphine and morphine was first effected by BOUGAULT (1902) who converted these alkaloids to the tartrates and then separated them as the sulfates, the sulfate of pseudomorphine being almost insoluble in cold water. DORLENCOURT (1913) precipitated both the alkaloids from urine as the silicotungstates. In order to regenerate the alkaloids from the silicotungstate combination, he treated the precipitate with sodium carbonate. The liberated alkaloids were then extracted with ammonia-amyl alcohol and the separation completed by transformation to the sulfates as in the Bougault method. GRIMBERT and LECLÊRE (1914) separated the mixture by precipitation of the pseudomorphine with potassium ferricyanide and sodium acetate in neutral solution. With their method it was possible to detect and isolate pseudomorphine even in a large excess of morphine. This precipitation method was about as sensitive as the silicotungstate method. BALLS (1926)(1926a) stated that the separation could be made by precipitating the free pseudomorphine base at its isoelectric point, or by adjusting the reaction of the mixed silicotungstates

with monobasic potassium phosphate to pH 7.2 to 7.5, whereupon pseudomorphine was completely removed as a mixture of silicotungstate and free base. With fairly large amounts of pseudomorphine the former procedure was satisfactory but with only a few milligrams, in the presence of proteins which act as protective colloids and interfere with the precipitation of the base, the alternative method was suggested. The morphine was then extracted at its isoelectric point, pH 8.9.

E. Pseudomorphine in the Organism

1. EXCRETION

GÉRARD, DELÉARDE and RICQUET (1905) were among the first investigators to claim the detection of pseudomorphine in urine of rabbits, only after acid digestion of the urine and extraction with ammonia-amyl alcohol solvent. DORLENCOURT (1913) also asserted that there was an excretion of pseudomorphine in the urine of rabbits injected intramuscularly with 150 mg. morphine hydrochloride per Kg. of body weight. The elimination was extremely small and was positive in all cases, but it was not possible to detect the quantities of pseudomorphine in each case.

2. IN TISSUES

GÉRARD, DELÉARDE and RICQUET (1905) claimed to have detected the presence of pseudomorphine in the kidneys and liver of a dog having received hypodermically 100 mg. morphine hydrochloride and then being sacrificed 6 hours after the injection. Acid hydrolysis of the tissues was necessary before the detection was accomplished.

F. Higher Oxidation Products of Morphine

In alkaline solutions, when shaken with air, pseudomorphine is gradually replaced by more highly oxidized compounds. Besides pseudomorphine, BALLS (1926) found that a large number of other substances, formed during the course of oxidation of morphine, were precipitated by phosphotungstic and silicotungstic acids and apparently as completely as morphine itself. These precipitants, if added to a partially oxidized morphine in solution under proper conditions, precipitate morphine, pseudomorphine and a heterogeneous group of substances resembling morphine and pseudomorphine in many ways. The precipitate was more readily soluble in water than that formed by morphine itself.

The higher oxidation products were partially precipitated by strong acids, and were completely and readily soluble in very weak alkali. On evaporation of the acid solution, these dark colored substances were partially soluble in the higher alcohols, chloroform and benzyl alcohol and nearly insoluble in the lower alcohols and ether. From alkaline solution these products were not removed by any of the solvents. These substances resembled morphine in their qualitative properties only by precipitability with most alkaloidal reagents, such as derivatives of tungstic, molybdic, and trichloracetic acids. They were more acid in character than either morphine or pseudomorphine.

In the BALLS' (1926) method for the separation of pseudomorphine as the silicotungstate at pH 7.3 to 7.5, neither the morphine nor its higher oxidation products interfered. The filtrate from the pseudomorphine precipitate contained the morphine and the other oxidation products, and they were separated by extracting the morphine from a solution of pH 9.0.

VI. CODEINE

A. Isolation

The problem of the isolation of codeine from tissues and body fluids is intimately associated with isolation procedures for morphine; references to its isolation are meager. Codeine is much more soluble than morphine in most organic solvents. It is far more stable toward oxidizing agents than morphine; it is unaffected in alkaline solution by oxygen, and does not give the reduction reactions which characterize morphine.

B. Excretion

1. URINE

SCHMEMANN (1870) reported positive tests for codeine in the urine of dogs after orally receiving 200 mg. per Kg. body weight. TAUBER (1892) also found that codeine was quantitatively excreted in the urine. Quantitative estimations for codeine in dogs' urine was reported by BOUMA (1903). One animal receiving subcutaneously 200 mg. codeine daily for 3 days; 300 mg. daily for 6 days, 400 mg. daily for 5 days with intermittent periods of 2 to 10 days between injections, yielded in the urine of those 3 periods 85.8, 80.2 and 84.5 per cent. of the injected doses. Daily collections of urine were made and continued for 2 days after the last injection. PANSE (1933) obtained a positive test for codeine from the urine of a patient receiving 500 mg. daily, as long as 6 hours after the last dose.

No codeine was found by NEUMANN (1893) in the urine of rab-

bits which had received single doses of 360 mg. codeine.

2. FECES

NEUMANN (1893) reported negative fecal eliminations from rabbits receiving single doses of 360 mg. codeine. BOUMA (1903) found the fecal output of dogs to be about 7 per cent. of all amounts subcutaneously injected.

C. Presence in Tissues and Secretions

1. TISSUES

OTOBE (1933) reported the presence of codeine in the brain of rabbits.

2. SECRETIONS

KWIT and HATCHER (1935) failed to detect even a trace of codeine in the milk of 5 women 2 to 4 hours after receiving a total of 130 to 192 mg. subcutaneously, some in 65 mg. doses 2 hours apart, others in 32 mg. doses every 4 hours.

D. Fate in the Body

The fate of codeine in the body is unknown. In the opinion of BOUMA (1903), the organism has no ability to destroy it.

VII. HEROIN

A. Isolation

The isolation procedures for heroin (diacetylmorphine) from tissues and excreta are identical with the procedures for the extraction of the previous 2 alkaloids. The ease with which heroin hydrolyzes makes the method for the extraction of this alkaloid with solvents less reliable than for the previous 2 alkaloids mentioned. McNally (1917) isolated heroin from tissues and claimed that his method of extraction entailed little decomposition and loss of alkaloid. The method involved the use of a weak acid in the extraction, low temperature for the concentration of the extraction fluids and the removal of the alkaloid by adsorption. The final aqueous concentrate, resulting from repeated alcoholic extractions and precipitations of extraneous matter, was shaken with a hydrated aluminum silicate (Alcresta) to adsorb the heroin. The adsorbed alkaloid was then removed by extracting with ammoniated chloroform in a separatory funnel. The isolated heroin was identified by color reactions.

1. QUANTITATIVE ESTIMATION

Since more sensitive methods are available for the determination of morphine than for heroin several investigators have recommended the conversion of heroin to morphine, and later determining the amount of heroin indirectly by the morphine method. ITO (1936) found that if a solution of heroin added to 2.75 per cent. sulfuric acid was heated to 100°C. for 50 minutes in a closed tube, a perfect reduction to morphine took place.

The biological method for alkaloid determination has been applied for heroin to a limited degree. MUNCH (1934) maintained that the mouse-tail curve reaction for heroin, in general, resembled that produced by morphine. In addition to this characteristic reaction, mice injected with heroin showed a series of symptoms differing from those following the administration of morphine; the most common one was the development of a definite running reflex. ITO (1936a) examined the relationship between the amount of heroin injected (mg.) for 1 gm. body weight (X) of the mouse and the duration period of tail-raising reaction (Y) and derived the formula:

$$Y = 3011.9\ X^{0.63094}$$

B. Excretion

1. URINE

LANGER (1912) was able to identify heroin, accompanied possibly by monoacetylmorphine, in the urine of a normal dog after it had received 120 mg. heroin subcutaneously. He also detected the presence of heroin in the urine of a rabbit which had been subcutaneously injected. This alkaloid has been qualitatively demonstrated in the urine of human addicts by PANSE (1933), TO and RIN (1933) and TO (1935). LANGER (1912) could not detect heroin in the urine of a dog which had received this alkaloid daily for 2 months.

2. FECES

LANGER (1912) found a small amount of an undetermined mor-

phine derivative in the feces of a dog which had received 120 mg. heroin subcutaneously. He could detect none in the feces of a dog which had received heroin daily for 2 months.

C. Presence in Tissues and Secretions

1. TISSUES

McNALLY (1917)(1917a) reported the presence of heroin in various organs of 2 individuals who died of poisoning from this alkaloid. The alkaloid found in the stomach and its contents, in one of the cases, responded to heroin tests and the remainder of the organs examined (liver, kidneys, spleen and intestines) gave positive tests for morphine. The same author recovered heroin in the heart, stomach, liver, kidneys, spleen, intestines and urine of 4 dogs poisoned with it, whereas a rabbit poisoned with 150 mg. heroin per Kg. body weight yielded positive tests for morphine in the various viscera.

2. SECRETIONS

Heroin was demonstrated by MUNCH (1934) in the saliva of horses, which had received 0.06 to 0.57 mg. heroin per Kg. by means of the mouse-tail reaction.

D. Destruction

1. IN SOLUTION

GORIS and FOURMONT (1931) claimed that heroin hydrochloride in cold aqueous solutions hydrolyzed with a loss, first, of one

and, finally, both acetyl groups after a period of several months. OBERST and ANDREWS (1941) found no appreciable change (0.1 per cent.) in the conductivity of heroin hydrochloride from 1 minute after the time of solution to 2 weeks after solution. They concluded that hydrolysis must proceed at a very slow rate.

2. IN THE ORGANISM

Little is known of the fate of heroin in the body but there are indications suggesting that it breaks down into monoacetylmorphine and then into morphine. When CLOETTA (1903) digested a normal rabbit's brain with 100 mg. heroin for 4 hours he was able to recover 71 per cent. of the added alkaloid. BABEL (1904) was able to recover 84 per cent. of 100 mg. heroin which was digested with the brain of a rabbit that had been receiving 250 mg. morphine daily for 8 months. McNALLY (1917)(1917a) reported that the alkaloid extracted from the liver, kidneys, spleen, intestines and intestinal contents of 2 human cases of heroin poisoning responded to all the reactions for morphine. The liver, bladder, urine and intestines of rabbits given heroin showed that deacetylation had taken place. The same author incubated for 3 hours, samples of fresh rabbit liver, heart and spleen with 100 mg. heroin hydrochloride and the alkaloid separated gave all tests for morphine. RIZZOTTI (1934)(1935) demonstrated that when a heroin solution was perfused through an active isolated frog's heart, the heroin was fairly rapidly converted into monoacetylmorphine and finally into morphine at a much slower rate. He found that heroin in contact with skeletal muscle, either contracting or resting, did not cause such a conversion. WRIGHT (1941) found

that the sera of some rabbits were able to remove only 1 acetyl group from heroin while the others were able to split off both acetyl groups "in vitro". Those animals able to remove the 1 acetyl group, split off only the more labile phenolic group while the others were able to form acetic acid by hydrolysis at both the phenolic and alcoholic positions of the molecule. Human sera deacetylated heroin at a much slower rate than rabbit sera. Wright found physostigmine to have a marked inhibiting action on the enzyme responsible for the hydrolysis. There were further indications that cholinesterase was not the enzyme responsible for removing the alcoholic acetyl radical and that 2 enzymes were concerned in the splitting.

VIII. CONCLUSION

The difficulty of extracting small amounts of morphine and its allied alkaloids from tissues, urine and blood has long presented a problem to the analyst. From the maze of published methods for isolation and determination of these alkaloids the selection of a suitable analytical procedure is unquestionably touch-and-go. The inability to extract small amounts of morphine in particular, from blood, urine and tissues serves to explain some of the contradictions recorded in the literature concerning the distribution of morphine in the body. Compilation of the data on control experiments for urine, a comparatively pure solution, shows that methods were developed to recover amounts of 4 mg. morphine, on the average, for 100 ml. urine with reported recoveries from 0 to 107 per cent. Similarly, for blood the methods were for 85 mg. per 100 ml., with recoveries from 84 to 101 per cent., which is a surprisingly large amount considering the traces of morphine sought in animal blood experiments.

Judging from the marked disparity in some of the results reported by the principal investigators, it is obvious, that in addition to faulty methods of analysis or experimental procedures, the presence of morphine in other forms and sources was overlooked. Under such conditions it is comprehensible why the biological estimations of the numerous investigators produced such varying results. The disregard of the kidneys as the most obvious route of morphine elimination explains the failure of the early workers to show the presence of morphine in the urine. The general complete neglect of the oxidized form of morphine as well as the inadequate

differentiation between morphine and its oxidation products is another definite source of error. In the recovery of the alkaloid from the tissues, some investigators failed to recognize the muscles as holding a much larger amount of the administered morphine than any other tissue. The review further reveals that there is no pronounced accumulation of morphine in the tissues for any prolonged period of time. The loss of morphine in the conjugated form, determined in the urine by some of the later investigators, casts further doubts on the earlier studies of the metabolism of the administered doses of morphine. Its fate can only be adequately determined by a comparative study of the amount given and the total amount excreted. Very little data on comprehensive studies of the metabolism of morphine is available. This is not surprising in view of the slowness, laboriousness and uncertainty of the methods for determining morphine.

The development of an accurate and fairly rapid method for the isolation and determination of minute amounts of morphine is, therefore, of primary importance for the solution of these problems.

Part II.

EXPERIMENTAL

ISOLATION AND DETERMINATION OF MORPHINE, CODEINE AND HEROIN FROM VISCERA AND BODY FLUIDS BY CHROMATOGRAPHIC ANALYSIS

SEPARATION OF MIXTURES OF MORPHINE, CODEINE AND HEROIN

EXTENSION OF THE CHROMATOGRAPHIC ANALYSIS FOR THE ISOLATION OF BARBITURATES

I. ISOLATION AND DETERMINATION OF MORPHINE, CODEINE AND HEROIN FROM VISCERA AND BODY FLUIDS BY CHROMATOGRAPHIC ANALYSIS

From the survey of the existing methods it is obvious that the degree of accuracy with which morphine and its allied compounds can be estimated, depend, not only on adherence to the precise conditions of a given method, but largely on the relative amounts of the alkaloid and the tissue and partly on the total amount of alkaloid to be determined. It is highly improbable that anyone can recover regularly a high percentage of morphine with the best reputed methods available when only 1 or even several milligrams are present in 100 ml. blood or 100 grams tissue.

An important objective was to find a method which avoided the particularised errors and attained a successful isolation of the alkaloids so that the morphine, codeine and heroin were purified nearly without loss. STEWART, CHATTERJI and SMITH (1937) focused the attention in the toxicological field on the possibility of adsorbing alkaloids on a solid medium. Of all the methods suggested or used this one seemed to offer the most hopeful line of attack. The immediate advantage of the adsorption method is that it lends itself readily to the isolation of extremely small quantities of chemical substances, thus eliminating the necessity for evaporation of large volumes of extraction media involving smaller losses than the conventional methods discussed in the first part of this thesis.

To apply this method effectively and successfully the main problem was to find a suitable adsorbing agent and to determine

the conditions required for the adsorption and elution of the alkaloids. No universal adsorbent has yet been found nor has a perfect adsorbent for any given purpose been developed. Notwithstanding the mass of information that has been published regarding the adsorptive abilities of various agents, the final selection of a suitable adsorbent for the specific alkaloidal separation still had to be made on a purely empirical basis. The theories of chromatography that have been developed have not made it possible to predict whether or not a given adsorbent can be used for the separation of a particular mixture of solutes. On account of the generally great variation in the adsorptive properties of solids prepared in the laboratory, a commercially prepared adsorbent is preferable. Such a solid with remarkably uniform adsorption properties was found in a commercial product, "Florisil." It was found to adsorb morphine very readily, and on the basis of this observation the columnar adsorption technique was applied for the development of a method for the isolation of morphine, codeine and heroin from viscera and body fluids. In the process of accumulating information on the adsorption of these alkaloids on the Florisil and its eventual elution, it became evident that mixtures of these alkaloids could be separated, either by selective adsorption or by selective elution.

It was the purpose of this work to attempt the isolation of 1 mg. or less of each of morphine, codeine or heroin from tissues and fluids. For such an isolation the use of adsorption columns offered some advantages. The unwanted constituents of the alkaloid-containing mixture could be removed either by selective adsorption of the alkaloid or by resolution of the alkaloid and im-

purities. Since these alkaloids are more stable in acid than in alkaline solution, at least when the temperature is above that of the ordinary laboratory, a primary consideration in the isolation was concerned with all operations including the adsorption being carried out, if possible, completely at reactions below pH 7.0.

A. Experimental

For the recovery of alkaloids in quantities of the magnitude of 1 mg. or less, all reagents including the adsorbents must be of a high grade of purity, i.e. they must be free from oxidizing agents and coloring material. All reagents used in these experiments were purified with the specific purpose of removing these interfering substances.

1. PREPARATION AND PURIFICATION OF MATERIALS

Florisil[1]

This is a synthetic magnesium silicate with a particle size of 60 to 100 mesh. (Standard U.S. Series Equivalent Sieves). It is a hard, porous, stable material of white granular appearance giving a pH of 9.8 when suspended in distilled water. It was purified by refluxing with a mixture of formic acid, ethyl alcohol and ethyl acetate.

Ethyl Alcohol

Each liter of 95 per cent. alcohol, laboratory grade, was

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1. Florisil was obtained from the Floridin Co. Inc. Warren, Penna., U.S.A. It is manufactured in accordance with U.S.Patent #2,393,625, and can also be obtained in sizes from 4 to 300 mesh.

mixed with 4 gm. silver nitrate dissolved in a minimum quantity of water and about 1 ml. 40 per cent. sodium hydroxide. After standing for 24 hours more sodium hydroxide was added until no further precipitation of silver occurred. The precipitated silver salt was removed by filtration and the alcohol refluxed for 1/2 hour and then distilled.

Methyl Alcohol

The same purification procedure as for ethyl alcohol was used.

Ethyl Acetate

Each liter of this reagent was refluxed for 1/2 hour with approximately 25 gm. P_2O_5 and then distilled.

Oxalic Acid

This reagent was purified by sublimation at temperatures between 140° to 157°C. after it had been rendered anhydrous by heating to 60°- 70°C. No special apparatus was required. The sublimation was carried out in a 1 or 2 liter glass-stoppered conical flask partially immersed in an oil bath at the stated temperatures. The sublimed product condensed on the cooler surfaces of the flask.

Formic Acid

Reagent grade of 90 per cent. formic acid was used.

Sodium Carbonate Solution

A saturated solution of the pure salt (Analar Reagent) was used.

Formaldehyde-Sulfuric Acid Reagent

1 ml. 40 per cent. formaldehyde solution was mixed with 99 ml. pure H_2SO_4 (sp. gr. 1.84).

Formaldehyde-Ferric Sulfate-Sulfuric Acid Reagent

0.2 ml. formaldehyde-sulfuric acid reagent was mixed with 60 ml. H_2SO_4 (sp. gr. 1.84) and then 8 ml. 10 per cent. aqueous ferric sulfate solution was added with cooling.

Phenol Reagent

Commercially prepared Folin-Ciocalteu reagent was used.

2. APPARATUS

Eluting Apparatus

In order to avoid the distribution of 1 mg. alkaloid in a large volume of solvent it was considered feasible to elute the adsorbed alkaloids by refluxing in a type of apparatus which would utilize a small volume of hot solvent. The following apparatus shown in Figure 1. was designed and fabricated by the author in this laboratory. A 100 ml. flask was used to contain the solvent. The lower ground glass joint can be either a B19 or B24; the upper one a B24 or B32. Any type of condenser can be used, the only stipulation being that the lower end has a protrusion centered over the funnel; this facilitates the flow of the condensed solvent into the adsorption tube. The stem of the inserted funnel is of sufficient length to enter the neck of the adsorption tube. So as not to hinder the return flow of the sol-

vent into the flask, 4 projections were made in the inner side-wall of the body of the apparatus to act as supports for the adsorption tube. The opening at the bottom of the body for the return flow is 1 to 1.5 mm. in diameter. This eluting apparatus worked very efficiently. Very little vapor came up through the bottom and at no time was there interference with the return flow of the liquid.

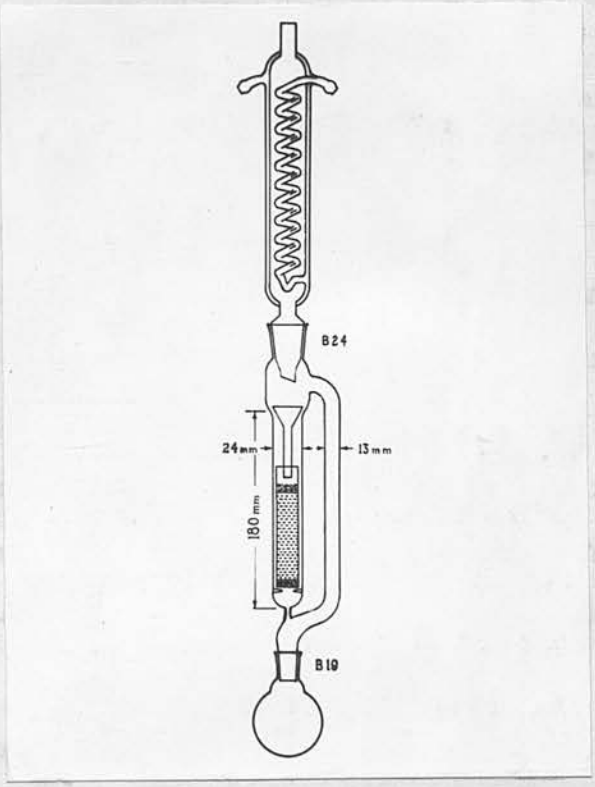


Fig.1

Adsorption Tubes

The tubes were made from pyrex glass tubing. Two sizes were used, 14 x 90 mm. and 14 x 120 mm. The former tube contained approximately 4 to 4.5 gm. Florisil and the latter 8 gm.

4. METHODS OF MANIPULATION

a. Preparation of Adsorption Columns

A satisfactory chromatographic analysis can be done with a simple apparatus. Only a few details will be elaborated upon since many of the methods of preparation of these columns and the general consideration of its use are excellently described by ZECHMEISTER and CHOLNOKY (1941), STRAIN (1942) and WILLIAMS (1946). For the preparation of the adsorption column a wad of cotton wool was firmly pressed into place at the bottom of the tube to act as a support for the Florisil. The adsorption tube was filled with the dry solid in 4 portions, each one packed down firmly with a plunger made of wood or metal. Only three-fourths of the tube was filled. To eliminate the disturbance of the top surface of the adsorbent in the column by the action of the dropping fluid a small wad of glass wool was placed on top.

The adsorbent packed into the column in this manner exhibited a uniform percolation of the solvent with a fairly rapid filtration rate without the aid of suction or pressure.

b. Refluxing of Columns

The preliminary cleansing of the adsorption columns and the elution of the alkaloid were both accomplished in the eluting apparatus. The procedure was simple. The cleaning or eluting solvent was boiled at a rate so adjusted as to assure a constant small layer of fluid above the Florisil. At times the percolation through the column was slow at the start, but it invariably increased to a satisfactory rate within a short time. In order to

detect the occasional occurrence of the sudden formation of an air lock in the top part of the column only a small layer of liquid which served as an indicator for the rate of percolation of the solvent through the column was permitted to collect above the Florisil. The certainty of the liquid percolating through the column was lacking when the adsorption tube above the adsorbent was filled to overflowing with the liquid.

In the procedures where the Florisil was overlaid with a salt necessary for the required elution, the salt was placed on the glass wool mat and then covered with a wad of cotton wool. The use of the latter was to prevent the drops of solvent falling on the salt and splashing it over the sides of the adsorption tube. It was not uncommon for an air lock to form between the salt layer and the adsorbent. This, however, was easily remedied by discontinuing the refluxing for a minute or two. The condenser was disconnected and a wire inserted through the funnel into the salt layer to break the air lock.

4. QUANTITATIVE METHODS

No attempt was made to develop new methods of determination for the 3 alkaloids. With small amounts as used in this experiment it was found expedient to use colorimetric methods.

a. Determination of Morphine

The OBERST (1939) method for the colorimetric estimation of morphine was used. The FOLIN-CIOCALTEU (1927) phenol reagent was substituted for the FOLIN-DENIS (1915) reagent. The morphine solution, transferred to a 100 ml. volumetric flask was alkalinized

with 20 ml. saturated sodium carbonate solution. 2 ml. phenol reagent were then added and the solution made up to volume. An intense blue color developed rapidly reaching maximal intensity within 2 hours and then remaining stable for many hours. All comparisons of the color were made after 2 hours in the photo-electric colorimeter using a red filter (Ilford 204). A rectilinear curve was obtained for concentrations of morphine from 0.1 to 2 mg. (Figure 2.).

A freshly prepared heroin hydrochloride solution in the same concentration as the morphine solution gives only a faint trace of a blue color with this reagent. On standing several days or longer the intensity of the blue color increases indicating hydrolysis of the heroin to give the free phenolic group. A pure codeine solution gives no color with the phenol reagent.

b. Determination of Heroin

With the formaldehyde-sulfuric acid reagent, heroin gives first a red color changing gradually to a reddish-blue. The dried residue obtained after the evaporation of the eluate was mixed with the reagent and stirred until all solid matter dissolved. The volume was then made up in a volumetric flask to 50 ml. with more of the reagent. The color stabilized within 1/2 to 1 hour and readings were made after 1 hour in the photo-electric colorimeter using the red filter (Ilford 204). The standard curve followed Beer's Law for low concentrations (Figure 3). Morphine and codeine give color reactions similar to heroin with this reagent as it is a non-specific reagent for alkaloids.

Fig. 2

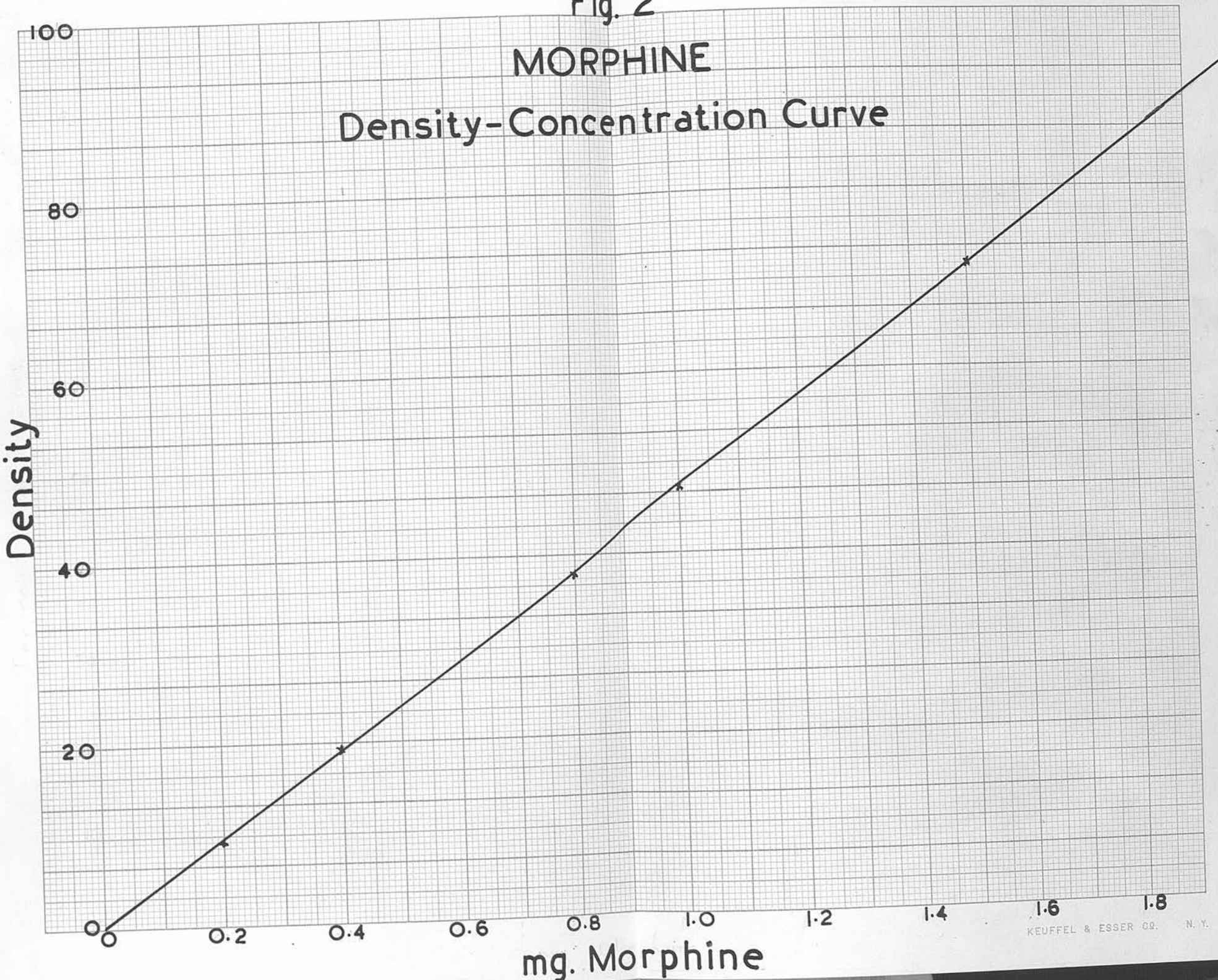

MORPHINE
Density-Concentration Curve
Density
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
mg. Morphine
KEUFFEL & ESSER CO. N. Y.

Fig. 3

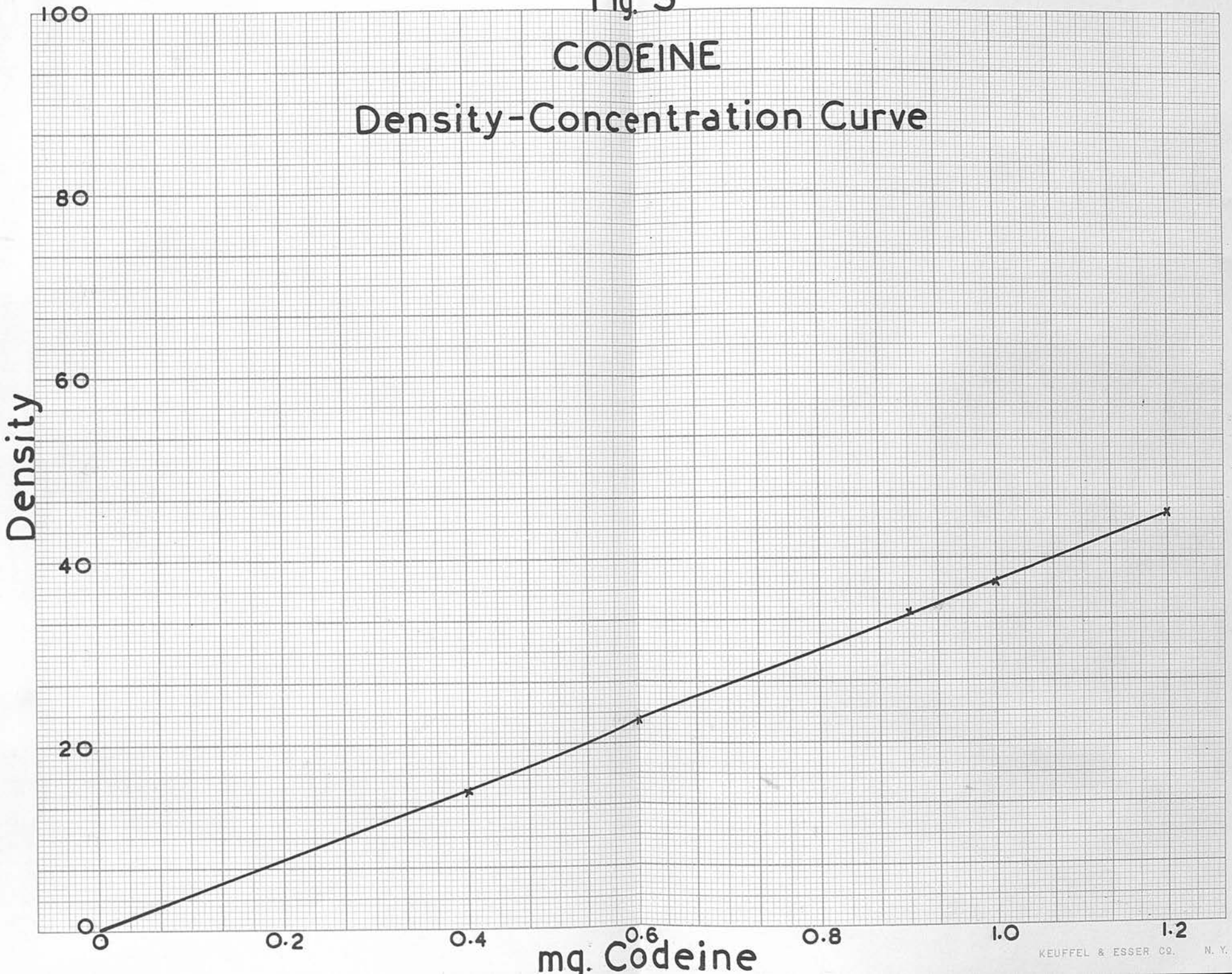
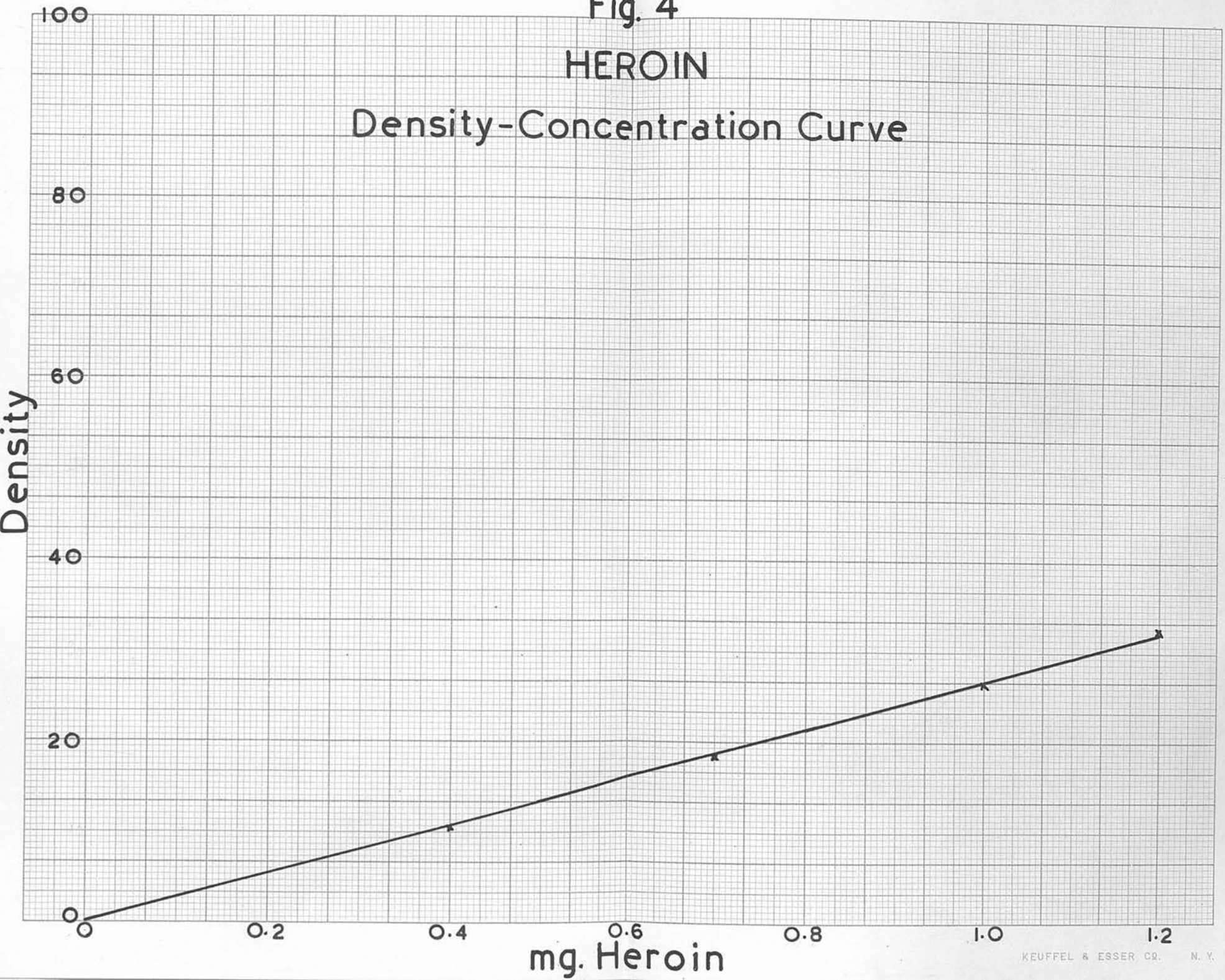

CODEINE
Density-Concentration Curve
Density
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
1.2
mg. Codeine
KEUFFEL & ESSER CO. N. Y.

Fig. 4

HEROIN

Density-Concentration Curve



c. Determination of Codeine

The formaldehyde-ferric sulfate-sulfuric reagent of Fulton (1929) serves as a convenient colorimetric reagent for codeine giving a reddish blue color. In the original reference 0.6 ml. formaldehyde-sulfuric acid reagent was suggested but 0.2 ml. of this reagent was found preferable. The method can be standardized with any quantity of formaldehyde reagent, but the blue color predominates with the lesser amounts of formaldehyde. The dried residue left by evaporation of the eluate was completely dissolved in some of the reagent. The volume was made up to 50 ml. with more of the reagent. The color was read after 1 hour in the photoelectric colorimeter using the red filter (Ilford 204). A standard curve was set up as for the other alkaloids within the limits of the concentrations used (Figure 4). In common with the heroin color reagent this is likewise a non-specific color reagent which gives colors with morphine and heroin, similar to codeine.

B. Development of the Method of Isolating the Alkaloids

Some preliminary experiments showed that Florisil possessed the capacity of adsorbing morphine without any preliminary treatment. Similar trials to determine whether it possessed similar adsorptive properties for codeine and heroin led to an expanding knowledge of adsorptive conditions necessary for these alkaloids.

1. ADSORPTION FROM WATER

Table 1.

Adsorption of Alkaloids from Water

Alkaloid	Untreated Florisil		"Treated" Florisil	
	Added mg.	Found mg.	Added mg.	Found mg.
Morphine	1.00	1.20	1.00	1.00
Codeine	1.00	1.05	1.00	1.00
Heroin	1.00	0.94	1.00	1.00

The adsorbent used without previous preparation is designated as the untreated Florisil. For "treated" Florisil each column was refluxed individually with approximately 50 ml. formic acid, ethyl alcohol and ethyl acetate mixture (1:3:3 by volume) for roughly about 2 hours. One milligram of the alkaloid in question was contained in 100 ml. water and run through the column. The amount of alkaloid adsorbed on the column was eluted by refluxing with methyl alcohol and determined quantitatively by the colorimetric methods already described.

With the yields of the eluted alkaloids recovered from the untreated adsorption columns one of the adsorptive properties of Florisil became manifest (Table 1). Of the three, heroin was not completely adsorbed on the untreated column, while the other two gave fictitiously high values. Since the alkaloids were adsorbed from a pure solution, this indicated the presence of impurities picked up from the column by the eluant.

The adsorptive property of the adsorbent with respect to heroin was augmented by the refluxing treatment with the mixture of formic acid, ethyl alcohol and ethyl acetate. No attempt was made

to determine the ratio of the 3 reagents for a constant boiling mixture. The amount of formic acid was increased to obtain a more acid column in later experiments. That the increased adsorptive activity was due to the refluxing solution or some constituent of the mixture was undeniable. The evolution of the refluxing mixture was based on experimental deductions. Refluxing with alcohol was used as a preliminary step for the removal from the Florisil of impurities which evidently interfered with the colorimetric determinations of the alkaloids. From some of the exploratory experiments for the adsorption of the alkaloids from salt solutions, ethyl acetate (later discarded) added to the solution appeared to cause an increased adsorption, i.e., the alkaloids were retained near the top of the column. The site of the retention of morphine on the column was obtained by extruding the adsorbent from the tube and painting it with solutions of ferric chloride and potassium ferricyanide. One milligram of morphine adsorbed on the column appeared as a deep blue band about 1 cm. wide at the top of the column. Ethyl acetate was added as a component of the cleansing mixture to increase the adsorptive capacity of the Florisil. Although this could not be definitely proved, the ethyl acetate was retained because, with the alcohol, it gave a column which produced no impurities that could be measured with the color reagents. The last component of the mixture, formic acid, was added for the purpose of acidifying the column and thus preventing the oxidation of the alkaloids retained by the alkaline Florisil; it was selected after various trials as the most suitable volatile acid with anti-oxidant properties.

2. ELUTION OF ADSORBED ALKALOIDS

The next objective, after complete adsorption of the alkaloids had been achieved, was the selection of an eluant for obtaining a rapid and complete liberation of the adsorbed materials. Of several solvents, such as ethyl alcohol, methyl alcohol, acetone, chloroform, ether, benzene and petroleum ether which were tried, the ethyl and methyl alcohols exhibited the required properties.

Table 2.

Elution Time of 1 mg. Adsorbed Morphine

Eluant	"Acid" Column	"Alkali" Column	Time of Elution hr.
Ethyl alcohol	0.0	1.00	1.0
Methyl alcohol	0.95	1.00	1.0
Methyl alcohol	——	0.97	0.5

Of the two, the methyl alcohol proved to be the better eluant. (Table 2) Whereas the ethyl alcohol only partially removed the adsorbed morphine from the acid column (acid from the acid refluxing treatment) even after prolonged refluxing the methyl alcohol removed it completely in an hour. In the "alkali" columns a layer of solid sodium bicarbonate or carbonate was placed above the Florisil in order that the hot alcohol could dissolve some of it and neutralize the acid in the column during the percolation process; under these conditions both alcohols gave complete elution in an hour. To obtain a complete recovery of the adsorbed morphine, the minimum elution time was detetmined as 45 to 60

minutes. It became apparent, however, that unless some means was devised to protect the eluted morphine contained in the boiling alcohol from the alkali that might percolate through the column, the destruction of the morphine was inevitable. A non-volatile alcohol-soluble acid, oxalic acid, placed in the flask containing the alcohol afforded the necessary protection.

In brief, the elution process was standardized for all the procedures by refluxing with 25 ml. methyl alcohol containing 0.5 gm. oxalic acid. The alcohol, before its passage through the column, percolated through a layer of 5 to 6 mm. sodium carbonate or sodium bicarbonate packed above the Florisil. After the elution period of 45 to 60 minutes, the alcohol solution was transferred to an evaporating dish or beaker. Distilled water in sufficient amounts was used to complete the transfer. The solution was placed on a water bath for evaporation to a small volume. For morphine the volume was reduced only to 10 to 15 ml. since its colorimetric determination was made in aqueous solution. For codeine and heroin, the solution was evaporated on the steam bath to the first signs of salt crystallization, never to complete dryness. For the final stage of evaporation, i.e., complete drying, the beakers were then placed in a warm air current (40-45°C.). Overheating on the steam bath is generally overlooked yet it is essential to avoid it, as heroin and codeine in minute quantities can be destroyed rapidly by excessive heat.

3. ADSORPTION FROM SALT SOLUTIONS

Adsorption of the alkaloids by the Florisil from aqueous, salt-free solutions served only to establish basic conditions for

adsorption and elution. The important problem of a possible salt effect, or presence of organic solvents such as are used in the extraction of alkaloids from solid matter, partially or completely inhibiting the adsorptive capacity of the Florisil, still remained. DAUBNEY and NICKOLLS (1938) were forced to discard adsorption and elution processes owing to the lack of adsorbing power of the materials used in the presence of comparatively large amounts of a strong electrolyte (ammonium sulfate). Although STEWART, CHATTERJI and SMITH (1937) found Fuller's earth and aluminium oxide relatively ineffective for the adsorption of morphine and strychnine from trichloracetic acid solutions, other substances tested, like kaolin, Franconite, Merck's medicinal charcoal, and aluminium oxide (Merck's "nach Brockmann") were found to be efficient in adsorbing relatively large amounts of alkaloids (10 mg. morphine, 50 mg. strychnine) from trichloracetic acid solutions.

To set up experimental conditions for the adsorption of these alkaloids from solution under conditions completely different from those which are obtained in the extraction of the alkaloids from tissue or blood would be at cross-purposes to the main objective, which was a direct extraction from such solutions. The adsorptive capacity of the Florisil for the 3 alkaloids having been established, it now remained to determine whether in the presence of salt, protein precipitating agents or alcohol, this capacity was affected. Trichloracetic acid solutions and aqueous-alcohol solutions with a minimum sodium chloride concentration of 1 per cent. were used for the duplication of conditions obtained in the extraction of tissues.

Table 3.

Adsorption of Morphine from Aqueous-Alcohol Solutions

Morphine mg.	H_2O ml.	C_2H_5OH ml.	CCl_3COOH gm.	$CH_3COOC_2H_5$ ml.	Recovery %
1.0	100	100	—	—	85.0
1.0	100	50	—	20	99.3
1.0	100	—	5	—	100.0
1.0	100	—	20	—	98.0

Table 4.

Adsorption of Morphine from Aqueous-Alcohol Salt Solutions

Morphine mg.	1% NaCl Soln. ml.	C_2H_5OH ml.	CCl_3COOH gm.	$CH_3COOC_2H_5$ ml.	Recovery %
1.0	100	75	5*	—	85.0
1.0	100	50	5*	—	92.0
1.0	100	50	5*	5	75.0
1.0	100	150	5	—	80.0
1.0	100	100	5	—	81.0
1.0	100	50	5	—	99.5
1.0	100	25	5	—	100.0
1.0	100	25	5	5	100.0
1.0	100	25	5	10	100.0
1.0	100	50	5	20	85.0
1.0	100	50	5	10	95.0
1.0	100(4%)	25	20	—	99.0
0.1	100	25	5	—	99.5
0.2	100	25	5	—	100.0
0.5	100	25	5	—	100.0
2.0	100	25	5	—	100.0
5.0	100	25	5	—	99.8

* not neutralized

In the preliminary experiments, trichloracetic acid solutions and aqueous-alcoholic solutions were used separately. Unless stated otherwise 1.0 mg. morphine was used in each adsorption experiment. An 80 to 85 per cent. adsorption of morphine was obtained from a solution in which the volume of alcohol was equal to

or greater than the volume of water. This indicated that the adsorptive property of Florisil was not seriously impaired by the presence of such a high concentration of alcohol. (Tables 3 and 4) Upon the reduction of the alcohol volume to half or less of that of that of the water the adsorption was complete. From a 5 per cent. trichloracetic acid solution which was neutralized with sodium hydroxide to about pH 7.0, a 100 per cent. adsorption of the added morphine was obtained. Proceeding to a 20 per cent. neutralized trichloracetic acid solution to determine whether a higher concentration of trichloracetic acid would affect the adsorption, a 98 per cent. retention was evident. The neutralization of the trichloracetic acid was found to be essential, since, when an unneutralized 5 per cent. solution of this acid percolated through the column, adsorption was incomplete (Table 4). This incomplete adsorption was subsequently determined to be due to a partial dissolving of the "magnesium silicate" by the action of the acid on the adsorbent since neutralization of the filtrate following such a percolation produced a gelatinous precipitate of silicate. These results are summarised in Tables 3 and 4.

Adsorption of the alkaloid from solutions containing both the alcohol and trichloracetic acid was considered useful. Such a mixture obtained from a tissue extraction would give a solution with a minimum amount of protein and protein breakdown products and would facilitate adsorption procedures. For the next step, investigation of the effect of the presence of salt, the trichloracetic acid concentration was, therefore, maintained at 5 per cent. and a 1.0 per cent. solution of sodium chloride replaced pure water. Trichloracetic acid solutions adjusted to pH 6.0-6.5

with varying amounts of alcohol gave complete adsorption as though each solution acted individually. An 80 per cent. adsorption was obtained when the alcohol volume was equal to or greater than the aqueous volume. The best adsorption was obtained from salt solutions when the alcohol volume was one-fourth the volume of the water and under these conditions, indeed, adsorption was complete. When the adsorption was made to occur at this definite alcohol concentration a high recovery of morphine (99 per cent.) was obtained even when the salt concentration was increased to 4 per cent. or the trichloracetic acid concentration to 20 per cent.

Exploratory experiments gave indications of ethyl acetate as well as isobutyl alcohol enhancing the adsorptive ability of the adsorbent as the result of a rough comparison of the columns after extrusion from the tube and painting with solutions of ferric chloride and potassium ferricyanide. These columns after the passage of the above-mentioned solutions containing ethyl acetate or isobutyl alcohol showed a more concentrated band of morphine at the top as compared to a slightly spreadout band after the passage of an ethyl acetate-free solution. Quantitative recoveries from such a solution showed that within certain limits up to 10 ml. ethyl acetate decreased the adsorption of morphine.

After the conditions for the maximum adsorption of morphine were established the recoveries for varying amounts of the alkaloid were finally determined. With the smaller sized column (14 x 90 mm.) complete recoveries from 0.1 to 2 mg. morphine were readily obtained. For the largest amount determined, 5 mg., it was found necessary to use a larger column (14 x 120 mm.).

4. DEPENDENCE OF ADSORPTION ON pH OF SOLUTION

Since the complete adsorption of heroin from pure aqueous solution had been found to be dependent somewhat upon the acidification of the adsorbent, it was necessary to determine to what extent the adsorbability of the 3 alkaloids varied with the pH of the solution percolating through the column when salts were also present. All solutions used for these determinations were 100 ml. aqueous solutions containing 1 per cent. sodium chloride, 5 per cent. trichloracetic acid and one-fourth its volume of 95 per cent. alcohol. Measurements of pH were made with the glass electrode. The Florisil columns, after the usual refluxing treatment, had pH's approximately between 7.0 and 7.5. In referring to the pH of the column, the pH measurement was that of about 50 ml. water passed through the column which had been previously washed by percolating 200 ml. water through it to wash out the acid retained from the refluxing mixture.

Table 5.

Adsorption of 1 mg. Alkaloid from Solutions at Different pH's

pH of Soln.	Morphine %	Codeine %	Heroin %
9.0	98.0	----	----
8.0	100.0	100.0	99.3
7.0	100.0	100.0	100.0
6.5	100.0	100.0	100.0
6.0	100.0	100.0	100.0
4.0	95.9	100.0	83.8
2.0	84.0	77.0	----

For all 3 alkaloids the best adsorptions were obtained from solutions whose pH's were within the limits of 6.0 to 7.0 (Table 5).

Morphine and codeine showed excellent adsorption over a wide pH range as compared to a slightly narrower pH range of adsorption for heroin. Above pH 8.0 and below pH 6.0 the adsorptions diminished. With the more acid solutions the decrease was accompanied by and possibly was due, mainly, to the partial dissolving of silicate by acid.

Adsorption from similar solutions within the mentioned pH ranges on more acid columns was less complete for morphine and codeine; while for heroin even less adsorption was evident. Acid columns of pH below 6.0 were obtained by longer treatment with the acid refluxing treatment or by increasing the formic acid concentration of the refluxing mixture.

C. Isolation of the Alkaloids Added to Tissue Extracts, Urine Filtrates and Blood Filtrates

In the isolation of the alkaloids from tissue, 2 main problems are involved. The first is the complete extraction of the alkaloid from the tissue and the second is its recovery from the extract in as pure a form as possible and the final determination of the amount recovered.

Many of the quantitative extraction experiments reported in the literature are of little or no value owing to the difficulty of obtaining the requisite condition, viz., the absorption of a known amount of alkaloid in the cells of a given quantity of tissue and the subsequent extraction of the alkaloid therefrom. As DAUBNEY and NICKOLLS (1937) pointed out, in determinations involving the addition of the alkaloid to the tissue so that the substance is largely extracellular, the degree of pulverization was

of little importance. When, however, the alkaloid had been absorbed into a tissue from the circulating blood and is, therefore, present within the individual cells, every cell must be ruptured to extract its contents; the degree of pulverization is then important. Some workers introduced new ideas for the complete rupture of the cells such as freezing of the tissue (DAUBNEY and NICKOLLS (1937)) and enzymatic digestion (TERUUCHI and KAI (1927) and FABRE (1924)). Improved tissue mincers or homogenisers which can equal the degree of pulverization produced by chemical or enzymatic methods have now been perfected. Such an homogeniser[1], demonstrated at the XVII. International Physiological Congress at Oxford, 1947, showed that no cell structure was evident after maceration of the tissue in the machine.

BALLS and WOLFF (1928) and DAUBNEY and NICKOLLS (1937) have given a good account of the various steps in the methods of mincing the tissue and extraction of the minced tissue. No further consideration will be given to the matter here as in the author's problem the primary aim was the quantitative isolation of the alkaloids from the fluids in the form of tissue extracts, blood filtrates and urine filtrates which had been obtained by means in general use. Only when that has been accomplished does it become possible to evaluate satisfactorily the various methods that have been proposed for the preparation of these fluids.

1. PREPARATION OF TISSUE EXTRACTS

Alcohol and aqueous trichloracetic acid tissue extracts obtained from animal livers were used in these experiments. For the

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[1] Manufactured by Nelco, Ltd., Shalford, Surrey, England

alcoholic extract small pieces of 100 gm. liver were macerated in a Waring Blendor with 200 ml. 95 per cent. alcohol. This mass was mixed with another 300 ml. alcohol and then filtered. The filtrate was clear and deep yellow in color. Each volume of filtrate was mixed with 4 volumes of 5 per cent. aqueous trichloracetic acid and filtered. The trichloracetic acid was used to precipitate some of the proteins dissolved by the alcohol and which, if allowed to remain, were partially adsorbed by the Florisil. The filtrate was ready for adsorption experiments after the addition of specified amounts of alkaloids.

For the preparation of the aqueous trichloracetic acid extract the method of STEWART, CHATTERJI and SMITH (1937) was used. Small pieces of 100 gm. liver were macerated in a Waring Blendor with 200 ml. 10 per cent. trichloracetic acid solution. The filtrate obtained was clear and light yellow in color. Each volume of filtrate was mixed with an equal volume of water in order to bring the concentration of the trichloracetic acid down to 5 per cent. For adsorption work, each 100 ml. of the 5 per cent. trichloracetic acid solution was mixed with 25 ml. 95 per cent. alcohol. The alcohol was found to prevent the complete adsorption of the impurities on the Florisil, and had, it will be recalled, been found to cause no interference with the complete adsorption of the alkaloids (Table 4).

2. PREPARATION OF BLOOD FILTRATES

Trichloracetic acid precipitation of the blood proteins produced a water clear filtrate. One volume of blood was mixed with 1 volume of a 10 per cent. aqueous solution of trichloracetic

acid and one-half volume 95 per cent. alcohol; i.e. for each 100 ml. blood, 100 ml. 10 per cent. trichloracetic acid and 50 ml. 95 per cent. alcohol were used. The coagulated proteins were removed by filtration and the filtrate used for adsorption experiments.

3. PREPARATION OF URINE FILTRATES

Deep yellow-colored normal human urine was used. As the urine contained a negligible amount of protein the use of trichloracetic acid was unnecessary. The urine was mixed with 1 volume of water and one-half a volume of 95 per cent. alcohol. The solution was filtered if necessary.

D. Recovery of Alkaloids Added to Tissue Extracts

The adsorption of alkaloids from tissue extract, blood filtrate and urine filtrate presented problems not encountered in the adsorption experiments previously discussed due to the presence of pigments, lipoids, residual soluble proteins and protein break-down products with their undetermined effects on the adsorption of alkaloids. The first problem was the partial or complete interference of alkaloidal adsorption and the second was the concurrent adsorption of the impurities with the alkaloids.

Preliminary trials of adsorption of morphine added to tissue extract, urine and blood filtrate with the conditions described for maximum adsorption from a pure solution gave only a small recovery of the added alkaloid. Since adsorption from the 3 extracts reacted in the same manner it was reasonable to assume that the conditions under which complete adsorption occurred from a

non-buffered solution did not apply equally well to "buffered" solutions such as those now being attempted (Table 6, line 1).

Table 6.

Adsorption of Morphine from "Buffered" Solutions

pH of Soln.	Non-Buffered CCl_3COOH %	Urine Filtrate %	Blood Filtrate %	Tissue Extract %
6.5	100	5.0	5.0	4.0
8.0	100	103.0	101.0	102.0

Good adsorption of morphine from the "buffered" solutions was obtained when the pH was adjusted to slight alkalinity for both the solution and column. (The term "buffered" is used with reservation until further experimental evidence shows that the differences in conditions for optimal adsorption for buffered and non-buffered solutions are due to some effect other than a buffering one). The Florisil columns were refluxed for a shorter period of time so that the column after the final washing gave a pH 7.5-8.0. The solutions or extracts were treated with sodium hydroxide solution to give a pH of 8.0. The high values of morphine recovered from the 3 extracts under these conditions (Table 6, line 2) showed that these impurities did not interfere with the adsorption of the alkaloid, although as anticipated some of the pigments were adsorbed simultaneously with the morphine. The columns with the adsorbed morphine were washed with alcohol-water mixtures but no attempt was made for the special removal of the last traces of impurities in these preliminary trials.

With the establishment of the optimal conditions for the ad-

sorption of morphine from the three mentioned extracts, the removal of the last traces of pigment and fat was accomplished by washing with 200 ml. of a mixture of water, alcohol and ethyl acetate in a ratio of 10:3:2 by volume. After such a treatment the column was completely colorless. The methyl alcohol, after elution of the column, was completely colorless but when the alcohol was transferred to the beaker for evaporation of the solution a slight cloudiness developed, this after evaporation of the alcohol formed a flocculation. The flocculation was due to proteins which precipitated as a result of the alkalinity of the adsorbent and dissolved again in the methyl alcohol. The flocculated proteins were successfully removed from the eluate by filtration through a tight wad of cotton wool and washing with 25 ml. water-alcohol (4:1) solution. At no time, after following such a procedure, was an "off color", especially with the sulfuric acid reagents, obtained for codeine and heroin. This procedure is applicable to any eluate in which proteins appear. The residue from the filtered alkaloidal-containing eluate, after being evaporated to dryness as required for the codeine and heroin determinations was colorless.

Table 7.

Recovery of Alkaloids Added To Tissue Extracts

Alkaloid	Amt. added mg.	Volume Tissue Extract ml.	Alkaloid Recovered mg.
Morphine	1.00	250	1.00
	0.50	200	0.50
Codeine	1.00	200	1.00
	0.50	250	0.49
Heroin	1.00	200	0.98
	0.50	250	0.48

Table 7. shows a few typical recoveries of alkaloids added, in amounts varying from 0.5 to 1.0 mg., to 200 ml. liver extracts. No blanks are recorded for the alkaloid-free tissue extracts. A blank determination using the phenol reagent for the color development gave only a trace of blue color which was equivalent to less than 0.01 mg. morphine. No equivalent comparison using the sulfuric acid reagents could be made for the blanks for codeine and heroin as the only color obtained was a light yellow color completely lacking any red or blue tint. In not a single case was a fictitiously high result obtained for any of the alkaloids when the columns were properly washed. In every single case where the sulfuric acid color reagents were used, colors identical with the colors from samples of pure codeine and heroin were obtained following correct washing of the columns as indicated in the technique. Recoveries of amounts of alkaloids less than 0.5 mg. were not attempted at the present time.

It is evident from Table 7. that within the range specified, the method gave results well within the limits of accuracy to be expected of an analytical process.

E. Recovery of Alkaloids Added to Urine Filtrates

The yellow pigment and the urea present in the urine solution were still factors to be reckoned with in the adsorption of the alkaloids on Florisil. A sample of the urine solution free of any alkaloid was run through a column, which was then cleaned by percolating through 150-200 ml. water-alcohol (1:4 by volume) solution. With the exception of a small amount of yellow pigment retained on top of the column, before washing, the urinary

pigments were not adsorbed. After washing with the aqueous alcohol solution only a trace of yellow was still visible near the top surface of the column. Elution of the column with methyl alcohol and various steps followed through for the development for a morphine reaction produced only a slight blue color which indicated only a small amount of impurity retained by the column. The same procedure was then repeated using a urine solution containing 1.0 mg. morphine. Over 100 per cent. recovery of morphine was obtained. The result was evidence that complete adsorption of the morphine had occurred and that neither the yellow pigments of the urine nor the urea present affected the adsorption to any appreciable degree. The fictitiously high result was caused by the simultaneously adsorbed pigments which were not removed by simply washing with a water-alcohol solution.

For the complete removal of the impurities adsorbed along with the alkaloid, the wash solution of the water, ethyl alcohol and ethyl acetate mixture previously found successful for washing of the column from the tissue extracts, proved as effective in this case. No trace of color was visible on the column after washing.

Table 8.

Recovery of Alkaloids Added to Urine Filtrates

Alkaloid	Amount added mg.	Volume Filtrate ml.	Alkaloid Recovered mg.
Morphine	0.5	250	0.50
	1.0	200	0.99
Codeine	0.5	250	0.49
	1.0	250	1.00
Heroin	0.5	250	0.48
	1.0	100	0.99

There was no difficulty, due to the precipitation of protein retained by the column and its subsequent elution with the methyl alcohol as in the case of tissue extract. The residues of the codeine and heroin on evaporation to dryness showed no visible traces of impurities and the colors obtained with the sulfuric acid reagents were identical with the colors from samples of the same amounts of these pure alkaloids.

Evidence of the accuracy of the recovery from urine is presented in Table 8.

F. Recovery of Alkaloids Added to Blood Filtrates

The adsorption of the alkaloids from blood filtrates offered none of the difficulties encountered with the lipoids of the tissue extracts or the pigments of the urine filtrates since they were free of both. The filtrate (approximately 225 ml.) from 100 ml. blood was used for each alkaloid determination. The trichloracetic acid was neutralized with sodium hydroxide and the solution was then adjusted to pH 8.0-8.5 before passage through the column.

Table 9.

Recovery of Alkaloids Added to Blood Filtrates

Alkaloid	Amount added mg.	Volume Filtrate ml.	Alkaloid Recovered mg.
Morphine	0.50	225	0.50
Codeine	0.50	225	0.49
Heroin	0.50	200	0.50

The recovery of only 0.5 mg. of each alkaloid was tried from blood filtrates. The accuracy of the procedure is shown in Table 9.

G. Techniques for the Isolation of the Alkaloids

The techniques finally adopted are set out in detail in this section. It was by their use that the figures given in Tables 7-9 were obtained.

1. FROM TISSUE EXTRACTS

A clear tissue extract was obtained either by the trichloracetic acid extraction method as described by STEWART, CHATTERJI and SMITH (1937) or the alcohol extraction method which forms the first stage of the classical Stas-Otto process. As the adsorption of the alkaloids was made from aqueous solutions containing 5 per cent. trichloracetic acid and one-fourth its volume of alcohol the necessary adjustments in the alcohol-water ratio in either solution were made before percolation through the Florisil column. The alcoholic extracts were mixed with 4 times their volume of 5 per cent. trichloracetic acid, the precipitate filtered off and washed with water-alcohol (1:4 by volume) solution. The pH of the solution was adjusted to 8.0-8.5 with sodium hydroxide solution. It was then passed through a Florisil column which had previously been refluxed with a formic acid-ethyl alcohol-ethyl acetate (1:4:4 by volume) for 2 hours and washed with 200 ml. water. Such a column gives a pH of 8.0 to 8.5. Following percolation of the total extract or a measured portion of it, the column was washed with 150-200 ml. water-ethyl alcohol-ethyl acetate (10:3:2 by volume)

solution. A slight amount of pressure, obtained from an ordinary aspirator bulb, was applied to the column to force out the remainder of the wash solution retained by the column.

The column was then removed from the percolation set-up, and the Florisil having been overlaid with solid pure sodium carbonate, was placed in the elution apparatus. Twenty-five ml. methyl alcohol containing 0.5 gm. oxalic acid were placed in the flask of the apparatus and at first heated slowly to assure percolation of the alcohol through the column without the formation of air-locks. As the rate of percolation increased the heating was increased. Total elution time was between 45 to 60 minutes. After the elution period was completed the alcohol was washed into a beaker with a sufficient quantity of water and the solution was evaporated on a water bath. When the volumes were reduced to about 15 to 20 ml., the solution was filtered if a flocculation of protein was evident, through a tight wad of cotton wool which was then washed with 25 ml. of a 4:1 (by volume) water-alcohol solution. For morphine determination the volume was finally reduced to about 10 to 15 ml. and for codeine and heroin determinations it was taken down nearly to dryness on the water bath with completion of the evaporation in a warm air current. The colorimetric determinations were then made on the residues.

2. FROM URINE FILTRATES

A volume of urine (100 ml. or more) was mixed with an equal volume of water (or 5 per cent. trichloracetic acid solution for urines containing large amounts of proteins). Alcohol equal to one-fourth the volume of the mixture, i.e. for each 100 ml. of

the mixture 25 ml. alcohol was added. The solution was filtered and the residue on the filter paper washed with alcohol-water (1:4) mixture. The solution was adjusted to pH 8.0-8.5 with sodium hydroxide solution.

The adsorption and elution of the alkaloids was then carried out exactly as described above for tissue extracts.

3. FROM BLOOD FILTRATES

Each 100 ml. blood was mixed with 100 ml. 10 per cent. aqueous trichloracetic acid solution and 50 ml. ethyl alcohol. The filtrate obtained from such treatment was adjusted to pH 8.0-8.5.

The procedure for the adsorption of the alkaloid on the adsorbent, removal of adsorbed impurities and elution of the alkaloid was identical with that used for tissue extracts and urine filtrates.

II. SEPARATION OF MIXTURES OF MORPHINE, CODEINE AND HEROIN

The separation and differentiation of the alkaloids has assumed a position next in importance to the isolation of the pure alkaloid from organic mixtures. For large amounts of alkaloids, the Stas-Otto method has been used to a limited degree for the separation of groups of alkaloids; for the identification of the alkaloids within the same group, its success depended upon some characteristic chemically reactive group of the alkaloid or upon some peculiar physiological property. Chemically, most of the alkaloids are characterized by color reactions. BAMFORD (1938) presented a systematic scheme for the differentiation of the more common alkaloids by classifying them according to color reactions which may also be used for their identification. Whether minute amounts of substances can be successfully subjected to such a scheme is highly questionable. By adsorption on columns of alumina and development of the chromatograms, KONDO (1937) was able to separate mixtures of morphine (upper band) and thebaine, of narcotine (upper) and thebaine, and of codeine (upper) and thebaine. The solvents used were either benzene or ether.

With the successful isolation of minute amounts of morphine, codeine and heroin in a pure form from organic mixtures attained by chromatographic analysis as presented in the first part of this experimental work, the problem of the possible separation of these 3 closely related alkaloids by the same means was examined. The first possibility for such a separation suggested itself when it was noted that by excessive acid refluxing of the Florisil its

adsorptive capacity was diminished.

Table 10.

Elution of 1 mg. of Alkaloid from "Acid" Columns

Eluant	Alkaloid		
	Morphine	Codeine	Heroin
C_2H_5OH	-	-	+
$KH_2PO_4 + C_2H_5OH$	-	-	+

Acid columns were obtained by refluxing for about 3 hours with the formic acid-ethyl acetate-ethyl alcohol mixture. The final 50 ml. of the 200 ml. water used for washing gave a pH 6.0 to 6.5. One milligram of the respective alkaloid was adsorbed from an aqueous 5 per cent. trichloracetic acid-alcohol (4:1) solution (pH 6.5). Ethyl alcohol was used for the elution of the adsorbed alkaloids, either alone or with solid monobasic potassium phosphate placed in a compact layer on top of the Florisil in the column. The acid phosphate was added so that an acid pH would be maintained during the elution process with the ethyl alcohol. The results obtained (Table 10) showed a decreased activity of the "acid" column for heroin, i.e., the plus sign indicated the elution of the alkaloid with ethyl alcohol, and the minus sign designated no elution. A possible separation of heroin from codeine and heroin from morphine therefore appeared likely. Nevertheless, actual trials with mixtures of 1.0 mg. each of morphine and heroin and 1.0 mg. each of codeine and heroin gave no such clear-cut separation. An occasional separation was obtained but the results were too eratic to be of any practical value.

A. Separation of the Alkaloids by Selective Elution

A second method of separation based on the elution of the adsorbed alkaloid with different organic solvents was attempted. The alkaloids were adsorbed on Florisil from aqueous 5 per cent. trichloracetic acid-alcohol (4:1) at pH 6.5. The Florisil was refluxed with mixtures of ethyl alcohol and ethyl acetate (1:1 by volume) for cleansing purposes.

Table 11.

Elution of 1 mg. Adsorbed Alkaloid

Eluant	Alkaloid		
	Morphine	Codeine	Heroin
Methyl alcohol	+	+	+
Ethyl alcohol	+	+	+
Ethyl acetate	-	+	-
Butyl alcohol	+	+	+
Acetone	-	+	+

The columns containing the adsorbed alkaloid were refluxed with the listed eluants (Table 11) for 30 minutes. Elution of the alkaloid is designated by a plus sign and non-elution (for the same period of refluxing) by a minus sign. Several eluants given in Table 11 appeared to be suitable for a possible separation of mixtures of several combinations of the alkaloids. When, however, mixtures of 1 mg. each of morphine and codeine and 1 mg. each of codeine and heroin were adsorbed on the column and then eluted with ethyl acetate (apparently the best eluant for their separation) and a mixture of 1 mg. each of morphine and codeine adsorbed

on a column and eluted with acetone, no separation but a complete elution of 2 alkaloids, was obtained. Separation of the adsorbed alkaloids on Florisil by elution with a selective eluant cannot be accomplished under the conditions of these experiments.

B. Separation of the Alkaloids by Selective Adsorption

These unsuccessful attempts at the separation of the alkaloidal mixtures at least indicated that these 3 alkaloids exhibited some differences in their adsorbabilities. A more promising approach to their separation appeared to be by a selective adsorption, i.e., by producing and maintaining conditions so that the more weakly adsorbed alkaloids are washed through the column. In the early part of this experimental work on the elution of the adsorbed morphine by refluxing with methyl alcohol, it was dis - covered that the Florisil, after such a treatment, lost its capacity to adsorb morphine again to any appreciable degree. When similarly treated columns were tried for adsorption of codeine and heroin (Table 12) no such inactivation for these 2 alkaloids was evident.

Table 12.

Adsorption of Alkaloids on Methyl Alcohol Refluxed Columns

Alkaloid	Percentage Adsorption	
	pH 6.0	pH 8.0
Morphine	1.2	3.0
Codeine	100.0	100.0
Heroin	100.0	98.0

The adsorption of morphine was nearly 2 per cent. greater from a solution at pH 8.0 than from one at pH 6.0, but was still almost negligibly small.

1. SEPARATION OF MIXTURES OF MORPHINE AND HEROIN

The separation of mixtures of morphine and heroin was based on the findings recorded in Table 12. Two columns were employed for the complete adsorption of mixtures of these 2 alkaloids. The first column was overlaid with sodium bicarbonate and refluxed with methyl-alcohol for 3 or 4 hours. After the refluxing period it was washed with 200 ml. water. The second column was refluxed with a formic acid-ethyl acetate-ethyl alcohol (1:4:4 by volume) mixture for about an hour. After washing with 200 ml. water and then 50 ml. water-alcohol (4:1 by volume) solution of which the pH was measured, the column gave a pH ranging between 7.5 and 8.0. The 2 alkaloids were placed in mixtures of 100 ml. of an aqueous trichloracetic acid solution and 25 ml. ethyl alcohol. The solution was adjusted to pH 6.0 and percolated through the methyl alcohol refluxed column, which was then washed by percolating 50 ml. water-alcohol (4:1 by volume) solution through it. The filtrate from the first column was adjusted to pH 8.0 to 8.5 and passed through the second column (formic acid refluxed), washed with 50 ml. water-alcohol (4:1) solution. Elution of the adsorbed alkaloids was made by the usual methyl alcohol procedure. In this way the heroin was completely adsorbed on and eluted from the first column, while the morphine was obtained on the second column. The extent to which the separation was attained is shown in Table 13.

Table 13.

Separation of Morphine and Heroin

	Mixture	Amount	Recovery	
			Heroin	Morphine
		mg.	mg.	mg.
1.	Morphine	1.0		0.96
	Heroin	1.0	1.00	
2.	Morphine	1.0		0.94
	Heroin	0.5	0.49	
3.	Morphine	0.5		0.49
	Heroin	1.0	0.99	

The largest recoveries were obtained with the alkaloid (heroin) which was adsorbed on the first column. The recoveries of morphine were well over 93 per cent. though rarely 100 per cent. As the heroin recoveries were never more than 100 per cent., it was unlikely that any of the morphine was retained in the first column since the sulfuric acid reagent for heroin gives a similar color with morphine. The adsorption of the morphine from the filtrate of the first column was unsuccessful under the conditions found suitable for its adsorption from trichloracetic acid solutions. After numerous trials, the optimal conditions for its adsorption were found to be identical to its adsorption from tissue extracts and urine and blood filtrates. The filtrate was, therefore, adjusted to pH 8.0-8.5 and percolated through a column of pH 7.5-8.0. The column was washed with water-alcohol (4:1) solution.

2. SEPARATION OF MIXTURES OF MORPHINE AND CODEINE

The method of separation of mixtures of morphine and codeine was identical to that used for the separation of mixtures of morphine and heroin. The heroin like the codeine was adsorbed on

the methyl alcohol treated column and the morphine was adsorbed, after its passage into the filtrate from the first column, on a formic acid-ethyl acetate-ethyl alcohol treated column. The conditions for its adsorption were identical with those described under the separation of mixtures of morphine and heroin.

Table 14.

Separation of Morphine and Codeine

	Mixture	Amount	Recovery	
			Codeine	Morphine
		mg.	mg.	mg.
1.	Morphine	1.0		0.94
	Codeine	1.0	1.00	
2.	Morphine	1.0		0.98
	Codeine	0.5	0.50	
3.	Morphine	0.5		0.50
	Codeine	1.0	1.00	

The separation of morphine and codeine can be considered as complete (Table 14). The recovery of less than the added amounts of morphine are probably due to some loss when the solutions were transferred for the adsorption of morphine.

3. SEPARATION OF MIXTURES OF CODEINE AND HEROIN

There were several indications that codeine and heroin could be separated from each other when they existed in a mixture although their adsorbabilities under the condition used thus far appeared to be nearly identical and an extension of the selective adsorption method used for the separation of morphine from codeine and heroin gave no promise of immediate success (Table 15). Nevertheless it was thought worthwhile to investigate the matter further.

Table 15.

Adsorption of Codeine and Heroin on
Treated Columns and from Solutions of various pH's

Treatment of Column	Percentage Adsorption			
	Heroin		Codeine	
	pH 6.0	pH 8.4	pH 6.0	pH 8.4
Acetone	98	100	100	100
Acetone & Formic Acid(4:1)	98	99	100	100
Methyl alcohol and Formic Acid (4:1)	99	100	99	100

The columns, refluxed with the mixtures containing formic acid (Table 15), were treated for a period (2-3 hours) sufficient to give a pH 6.5-7.0 after washing the column with water. The adsorption of both alkaloids was identical and this line of investigation was temporarily abandoned.

Reconsideration of the factors which were responsible for the shifting of the adsorption of the morphine from pure solution in the acid range to the alkaline range for "buffered" solutions like urine filtrates, etc. (Table 6) indicated a possible approach for the separation. As stated under the separation of heroin and codeine from morphine, after the solution containing the morphine had percolated through the methyl alcohol treated column the morphine then had to be adsorbed from an alkaline solution on an "alkaline" column. This was similar to the conditions established for the removal of the morphine from tissue extract, blood or urine filtrates. The three alkaloids reacted identically when adsorbed from pure aqueous trichloracetic acid solutions containing alcohol.

Table 16.

Adsorption of 1 mg. of the Alkaloids
on "Acid" Columns (pH 6.5) and from Acid Solutions (pH 6.5)

Solution	Percentage Adsorption		
	Morphine	Codeine	Heroin
Trichloracetic Acid	100	100	100
"Buffered"	100	100	100
Silicate	0	0	0
Tissue Extract	0	0	0
Urine Filtrate	0	2	100

All of the solutions listed in Table 16 contained alcohol in the ratio of one part to 4 parts of the aqueous solution. All the solutions with the exception of the urine filtrate contained 5 per cent. trichloracetic acid. The "buffered" solution contained a phosphate buffer. The silicate solution was obtained by percolating 100 ml. alcohol-water trichloracetic acid solution at about pH 4.0 through an untreated Florisil column. The filtrate was adjusted to pH 6.5, the alkaloids added and the solution then percolated through the "acid" column. The urine filtrate was prepared by mixing one volume of urine with an equal volume of water and one-half volume of 95 per cent. alcohol.

The differences in the adsorbability of the three alkaloids from the urine filtrate suggested a means of separating a mixture of codeine and heroin into its individual components and an alternative method of separating a mixture of heroin and morphine into its individual alkaloids. Experiments using urine filtrates for the separation of codeine from heroin and morphine from heroin failed, however, to produce such a selective adsorption of heroin on the acid columns as was to be expected from Table 16. In each

attempt, both the alkaloids in the mixture were adsorbed on the acid columns. It could only be assumed that this variation in the adsorbabilities was influenced by some undetermined factor.

C. Mutual Interference of Alkaloids on Adsorption

The columnar adsorption procedure for the isolation of a single alkaloid from an extract or the separation of 2 alkaloids into their individual components from a pure solution appeared to be simple and straight-forward up to this point. When the separation of heroin from codeine from complex organic mixture was tried some other factor or substance played a rôle not previously encountered. It could either be a trace of protein, a protein break-down product or a mutual interference of one alkaloid on the adsorption of the other in the presence of a third substance like proteins or silicates. The adsorption of mixtures of the three combinations of the three alkaloids from tissue extracts and silicate solutions is shown in Table 17.

Table 17.

Mutual Interference of Alkaloids on Adsorption from Tissue Extracts and Silicate Solutions

Alkaloid	Amount mg.	Adsorption from Solutions Silicate Soln.	Tissue Extract
1. Heroin & Codeine	1.0 + 1.0	- -	
Heroin & Codeine	1.0 + 1.0		+ +
2. Morphine & Codeine	1.0 + 1.0	+ +	
Morphine & Codeine	1.0 + 1.0		+ +
3. Morphine & Heroin	1.0 + 1.0	+ +	
Morphine & Heroin	1.0 + 1.0		+ +

The columns used in these experiments (Table 17) were treated in the usual manner to obtain a pH of 6.0-6.5. All solutions were adjusted to approximately pH 6.5. The silicate solution was the same as that used in the experiments described for Table 16. The double signs are listed to indicate that either both alkaloids were adsorbed (+ +) or not adsorbed (- -) on the acid columns.

When the individual alkaloid (Table 16) was present in either the tissue extract or silicate solution no adsorption was evident under the conditions stated. On the other hand when a second alkaloid was present (Table 17), with the exception of the heroin-codeine mixture in silicate solution, adsorption of both took place.

The few results reported on the mutual interference of alkaloids are as far as the present work on adsorption of the alkaloids has been developed. Their importance lies in their demonstration that the adsorption method, excellent though it has proved to be for the isolation and identification of single alkaloids of the morphine group, cannot safely be used in circumstances which involve the possible presence of two or more alkaloids of this group. If a mixture is known to be present, a partial separation is possible, but so far, it has not been possible to determine separately heroin and codeine present together. This suggests further, that for extension of the adsorption method to other alkaloids, an obviously reliable development of the method must involve much work in which the problem of mutual interference must be considered. This, however, is for the future and is an extension of the immediate problem, the determina-

tion of morphine, codeine and heroin, which is the subject of the present thesis.

III. DISCUSSION

The problem of this thesis was specifically the isolation of morphine, codeine and heroin in a pure form from tissue extracts containing lipoids, proteins, protein break-down products and pigments, and from highly pigmented urines. The isolation of these alkaloids added to tissue and blood itself was not undertaken at the present time because the adsorption, retention or alterations of the alkaloids in the cells is one of many problems which depend for their solution primarily on the quantitative isolation and determination of these alkaloids from tissue extracts and blood filtrates. The isolation of these alkaloids from tissue extracts and similar solutions by the usual methods of immiscible solvent extraction has not been accomplished with any real degree of success for the amounts of morphine, codeine and heroin of the magnitude worked on in this problem. Yet it is only when success has been achieved in this isolation that the further problems of distribution, metabolism, etc. can be attacked with reasonable hope.

Magnesium silicates have been used before for adsorption of alkaloids from aqueous solutions. MUTCH (1936)(1937) listed a series of silicates of magnesium, natural and synthetic, and demonstrated that synthetic hydrated trisilicates of magnesium exhibited powerful adsorbent qualities. Distilled water left in contact with trisilicate of magnesium (Mutch) acquired a pH of about 8.5 which was less alkaline than that of Florisil (pH 9.8) in contact with water. Many alkaloids were removed from pure solution by this trisilicate and a selective affinity for certain alkaloids

was discernible. Many colloids, for example, starch, whey proteins, proteoses, "acid albumen" and "alkali albumen" prepared from egg white were also removed from solution. Although its immediate adsorptive capacity is considerable Mutch found that several days were required for saturation of the trisilicate, within which time as much as 11 mg. morphine hydrochloride and 15 mg. codeine sulfate respectively were removed by 1 gm. of the silicate.

The apparently small amount of morphine (less than 5 mg.) adsorbed by about 5 gm. Florisil is in agreement with the Mutch observation as the rate of percolation of the liquid through the column was relatively rapid (approximately 200 ml. water per hour).

Under the conditions given, Florisil exhibited a smaller adsorptive capacity for the pigments than it did for the alkaloids. The lipoids did not appear to be adsorbed due to the ease with which they were washed through the column. On the other hand, some proteins, from the tissue extracts, in particular, were retained by the column. Their retention, without becoming involved with the mechanics of the reaction of adsorption, was considered to be due to an adsorption of proteins on the column in spite of the alkalinity of the latter. The proteins thus retained in the column were aqueous-alcohol soluble and subsequently appeared in the methyl alcohol eluate.

None of the objections to the extractive methods recorded in the literature are applicable to this method. The identical procedure for adsorption and elution of the alkaloids can be used for any extract or filtrates.

The results reported represent the work accomplished with

several hundred columns not inclusive of several times that number for the establishment of the optimal conditions for adsorption of the alkaloids from water solutions, aqueous-alcohol salt solutions, tissue extracts, urine and blood filtrates and for the separation of mixtures of the alkaloids. The values stated are typical, not average, values of the many recoveries from the solutions and filtrates described.

The separations of mixtures of the alkaloids were not accidental but were accomplished as the result of experimental evidence gained regarding their differences in adsorbabilities under varying conditions. The adsorbent, after treatment with methyl alcohol, showed a marked difference in its adsorptive power for morphine only from pure solutions. CHRISTENSEN (1945) reported a similar experiment in which methyl alcohol inactivated a highly adsorptive aluminium oxide for the adsorption of procaine hydrochloride. The separations as reported are from pure solutions. Any variation of the stated conditions may produce entirely different results.

At the present time no differences have been observed in the adsorbabilities of codeine and heroin from pure solution although this does not exclude the possibility that such a difference may be found under other conditions which will permit the required separation.

The discovery of the mutual interference of alkaloids on adsorption from tissue extracts and silicate-containing solutions should lead to some future interesting experimental work.

IV. EXTENSION OF THE CHROMATOGRAPHIC ANALYSIS FOR THE ISOLATION OF BARBITURATES

After the successful isolation of morphine, codeine and heroin by adsorption on Florisil it was decided to extend the method and to determine under what conditions some of the other frequently encountered drugs, particularly the barbiturates, could be separated from these alkaloids, isolated and determined. A number of experiments with Barbital (diethylbarbituric acid) and Phenobarbital (phenylethylbarbituric acid) on "alkaline" and "acid" Florisil columns from acid or alkaline solutions similar to those from which the three alkaloids had been adsorbed, showed that there was no adsorption of these barbiturates. From such experimental evidence it appeared possible to separate a mixture of these alkaloids and barbiturates.

The removal of the barbiturates from eluate of the Florisil column by adsorption on another solid was attempted. Many otherwise possible solids were eliminated on the basis of their fine particle size which made them very inconvenient for use in an adsorption column due to its slow percolation rate. Activated carbon with its well known adsorption capacity for many substances, among them the barbiturates was, therefore, tried for the removal of the barbiturates from the water-alcohol-trichloracetic acid solution. Cocoa-nut shell charcoal chosen both for its adsorptive capacity and its particle size, among a number of charcoals tried, was found to be the most suitable for this specific problem.

At various times charcoals have been advocated either for the adsorption of barbiturates from impure solution or for the adsorption of the impurities. For the former process BRUNDAGE and

GRUBER (1937) adsorbed the barbiturate and pigments on activated carbon (Norit A) and by selective elution with a mixture of equal amounts of ether and petroleum ether removed the barbiturate only.

Charcoal has, however, been chiefly used for the adsorption of pigments from impure barbiturate solutions. FRERICHS and FRERICHS (1906), FABRE and FREDET (1925) and BRÜNING and KRAFT (1927) purified the final aqueous barbiturate extract by adsorption of the impurities on charcoal. Fabre and Fredet claimed that none of the barbiturates were adsorbed by the charcoal. Cohen (1946) purified the chloroform extract of the barbiturate with charcoal.

A. Experimental

All reagents were of the highest purity obtainable or were purified to obtain the necessary grade of purity.

1. PREPARATION AND PURIFICATION OF MATERIALS

Charcoal

The most suitable charcoal for the adsorption of barbiturates was found to be activated cocoa-nut shell charcoal obtained from civilian gas mask. It was ground down to 60-100 mesh size. The individual charcoal column was purified by refluxing with ethyl acetate for 2 hours in the eluting apparatus previously described. At the completion of this purification the column was removed from the apparatus, the ethyl acetate retained in the column was forced out by application of a little pressure and the column was then refluxed for 1 hour with ethyl alcohol. Before use the column was washed with 100 ml. water.

Ethyl alcohol

This solvent was purified as stated under alkaloids.

Absolute Methyl alcohol

This solvent was purified as stated under alkaloids. It was then made anhydrous by treatment with freshly heated calcium oxide and distillation over the calcium oxide.

Ethyl acetate

Purification procedure is listed under alkaloid.

Chloroform

The purest grade of anaesthetic chloroform was used.

Ether

Freshly distilled peroxide-free ether was used.

Isopropylamine Reagent

A 5 per cent. isopropylamine solution in absolute methyl alcohol was used.

Cobaltous Acetate Reagent

A 1 per cent. cobaltous acetate solution in absolute methyl alcohol was used.

2. APPARATUS

The apparatus described for the isolation of the alkaloids was also used for the isolation of the barbiturates. Adsorption tubes, 14 x 120 mm. were used.

3. METHODS OF MANIPULATION

For the preparation of the adsorption columns of charcoal and refluxing of these columns the same technique was used as in the preparation of the Florisil columns. No difficulty was encountered with air locks on refluxing with these columns.

4. QUANTITATIVE METHOD

The color reaction produced by the interaction of the barbiturates with a cobaltous salt in an alkaline medium is not specific. It became apparent that from the studies reported the final color reaction could be obtained regardless of the type cobaltous salt or alkalinizing agent used. KOPPANYI, DILLE, MURPHY and KROP (1934) recommended that the isopropylamine cobaltous acetate color reaction be employed for semi-quantitative estimations of the barbiturates. This colorimetric method as modified by LEVVY (1940) was used in this work.

Quantitative estimations for 5 mg. of barbiturate were made by dissolving the barbiturate in 2-3 ml. chloroform adding 0.2 ml. cobaltous acetate solution and 0.6 ml. isopropylamine solution. The final volume was made up to 5 ml. with chloroform. The bluish pink color developed immediately and was stable for more than one-half hour. Comparison of the colors were made within one-half hour of the development of the color in the photoelectric colorimeter using a blue-green filter (Ilford 302).

For the estimation of 1 mg. barbiturate the same quantities of isopropylamine and cobaltous acetate reagents were used but the final volume was made up to 2 ml. with chloroform.

It was not possible to employ one barbiturate as a standard

for the quantitative estimation of the other barbiturates as the various barbiturates did not produce the same amount of color per unit concentration.

B. Development of the Method of Isolating the Barbiturates

It has already been established that the barbiturates were not adsorbed on Florisil columns. The conditions under which they are adsorbed on and most readily eluted from the charcoal now had to be determined.

1. ADSORPTION FROM WATER-ALCOHOL-TRICHLORACETIC ACID SOLUTION

To match the eluate of the Florisil columns, solution containing the same quantities of alcohol, trichloracetic acid and salt were used.

Table 18.

Adsorption of Barbiturates from Acid and Alkaline Water-Alcohol-Trichloracetic Acid Solutions

Barbiturates	Amount added mg.	pH 5.5 found mg.	pH 8.5 found mg.
Barbital	1.00	1.00	0.98
	5.00	5.00	5.00
	5.00*	0.00	0.00
	5.00**	0.00	0.00
Phenobarbital	1.00	0.99	0.98
	5.00	5.00	5.00

*Solution contained 5 ml. ethyl acetate per 100 ml.
**Solution contained 5 ml. amyl alcohol per 100 ml.

The recoveries from pure aqueous alcohol solution are shown in Table 18. The adsorption was complete either at pH 5.5 or pH 8.5.

The ethyl alcohol, in amounts used for the adsorption of the alkaloids, did not interfere with the adsorption of the barbiturates. Other organic solvents, such as ethyl acetate or amyl alcohol, even in small quantities, when added to the solution completely prevented the adsorption of the barbiturates.

2. ELUTION OF ADSORBED BARBITURATES

Many organic solvents readily removed the barbiturates from the charcoal. The one of choice was ethyl acetate as it removed none of the impurities from the charcoal itself. No special precautions were necessary to protect the barbiturates during the elution process since they were stable as indicated by the color reactions. A minimum elution period of 1 hour was found to be essential for the complete removal of 5 mg. adsorbed barbital. Elution for 30 minutes gave only a 95 per cent. recovery.

The elution process simply consis ted of refluxing with about 20 ml. ethyl acetate for 1 hour. The eluate was transferred to a beaker with the aid of a sufficient quantity of water. The solution was then evaporated on the water bath to dryness. The residue was dissolved in 15 ml. hydrochloric acid-acidified water. The solution was extracted 3 times with 10 ml. quantities of ether. The ether residue on evaporation was colorless and crystalline. The crystals were dissolved in chloroform for the colorimetric determination.

C. Isolation of Barbiturates Added to Tissue Extracts, Urine Filtrates and Blood Filtrates

In the first attempts to isolate the barbiturates from tissue extracts, urine and blood filtrates, the barbiturates were

added to the filtrates of the Florisil columns.

Table 19.

Recovery of Barbiturates Added to Tissue Extracts, Blood Filtrates & Urine Filtrates

Barbiturate	Amount added mg.	Solution used	pH of Soln.	Barbiturate found mg.
Barbital	5.0	Tissue	8.5	2.30
	5.0	Tissue	5.5	4.98
	1.0	Tissue	5.5	1.00
	1.0	Blood	5.5	0.99
	5.0	Blood	5.5	4.99
Phenobarbital	1.0	Tissue	5.5	0.97
	5.0	Tissue	5.5	5.00
	5.0	Tissue**	5.5	4.98
Barbital	1.0	Tissue**	5.5	0.96
	5.0	Tissue**	5.5	4.95
	5.0	Urine*	5.5	—

*Not estimated
**Solutions containing morphine and barbiturate percolated through a Florisil column

Unlike the pure solution, the tissue extracts gave poor recovery of the barbiturate from the alkaline range (Table 19). At pH 5.5 almost complete recoveries were obtained from all the solutions. Very little loss of the barbiturate was found to take place on passage through the Florisil column. Crystalline barbital was obtained from the ether residue of all the solutions tried including the urine filtrates. No quantitative estimation of the barbiturate in urine was attempted at this time. On percolation through the charcoal column of the urine filtrate containing the pigments, nearly all the pigments were adsorbed. On elution of the charcoal with ethyl acetate only a slight amount of these pigments was removed with the barbiturates. The slight amount of

the pigment also extracted by the ether did not interfere with the crystallization of the barbiturate but did interfere with its colorimetric estimation.

All filtrates from the Florisil column, that were to be used for the adsorption of barbiturates, had to be removed before the final washing of the Florisil column with the water-ethyl alcohol-ethyl acetate solution. Ethyl acetate as well as amyl alcohol in quantities as small as 5 ml. per 100 ml. solution prevented the adsorption of both the barbiturate and the pigments. (Table 18). The evidence from this experimental work indicates that the barbiturates were adsorbed with the impurities and when a reagent was added to hinder the adsorption of one it acted similarly on the other.

The work on the adsorption of barbiturates reported in this paper is only of a preliminary nature. It could form the groundwork to an extension of the adsorption analysis for future work. It is in no way to be construed as a completed analysis. More barbiturates, especially the unstable ones, need to be determined by this method and some means has to be found to purify the barbiturates extracted from urine, for at the moment, good recoveries have been proved only for tissue extracts and blood filtrates which do not contain the pigments which, in the case of urine, interfere with the colorimetric determination.

V. SUMMARY

1. Morphine, codeine and heroin in amounts of 0.5 to 1.0 mg. were adsorbed quantitatively from pure water-alcohol (1:4 by volume) solutions containing from 5 to 20 per cent. trichloracetic acid and from 1 to 4 per cent. sodium chloride. The adsorption was made on an acid column (pH 6.5 to 7.0) from acid solutions (pH 6.5).

2. The three alkaloids were adsorbed quantitatively from tissue extracts, blood filtrates and urine filtrates only on alkaline columns (pH 7.5-8.0) from alkaline solutions (pH 8.0-8.5).

3. Mixtures of morphine and heroin in pure solutions were separated into the individual components by selective adsorption of the heroin on methyl alcohol treated columns.

4. Separation of mixtures of morphine and codeine in pure solution was also accomplished by selective adsorption of the codeine on methyl alcohol treated columns.

5. The separation into the individual components of mixtures of codeine and heroin has not been successful under the conditions described.

6. Evidence for the mutual interference of alkaloids on adsorption from acid tissue extracts or silicate-containing solution on acid columns was given.

7. The adsorption method was extended for the separation of barbiturates from the alkaloids.

8. Barbiturates were adsorbed quantitatively in amounts from 1.0 to 5.0 mg. on activated charcoal from pure water-alcohol trichloracetic acid solutions either at pH 5.5 or pH 8.5.

9. Barbiturates were adsorbed quantitatively from tissue extracts, blood and urine filtrates only in the acid range (pH 5.5).

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