dilute solution of sodium carbonate added until alkaline. The yellow color of the solution changed to deep brown and on cooling yellow silky needles of the free alkaloid were obtained. They were purified by repeated crystallizations from hot water and finally from hot alcohol. (Yield, 10 Gm.)

The alkaloid melted at 205–207° C. (decomp.). Repeated crystallizations of the alkaloid did not change its melting point. The results of combustion analyses conform to the empirical formula, $C_{21}H_{21}O_8N$.

Analysis.—Calculated for $C_{21}H_{21}O_8N$, $^1/_2H_2O$: C, 59.4; H, 5.2; N, 3.3. Found: C, 59.0; H, 5.3; N, 3.3.

Umbellatine mixed with the alkaloid did not depress the melting point of the latter. Mixed melting point of nitrosoumbellatine and nitroso derivative of the alkaloid remained unchanged at 265–267° C. (decomp.). The amount of platinum in the chloroplatinate was found to be 15.63%, whereas platinum present in umbellatine chloroplatinate was determined as 15.71% (12). Calculated for $(C_{21}H_{21}O_8N,HCl)_2PtCl_4$: Pt, 15.72%. The coincidence of these results led me to conclude that umbellatine is present in *B. insignis*, Hook. f.

Umbellatine Sulfate.—Umbellatine sulfate is prepared by the usual method. Samples obtained from both sources melt at 274° C. (decomp.).

Umbellatine Picrate.—On treating a solution of umbellatine in alcohol, with an alcoholic solution of picric acid, the picrate was precipitated at once. The picrate was crystallized from hot alcohol as brown feathery needles. It melts at 232° C. (decomp.).

Analysis.—Calculated for $C_{27}H_{24}N_4O_{15}$: N, 8.7; Found: N, 8.9.

SUMMARY

1. Berberine and associated alkaloids were found to be present in many species of *Berberis*. Himalayan *Berberes*, however, showed the absence of berberine and yielded a different alkaloid.

2. One of the Himalayan *Berberes*, *B. insignis*, Hook. f., contains 1.0% of total alkaloid.

3. An alkaloid has been isolated and is found to be identical with umbellatine, $C_{21}H_{21}O_8N$, the alkaloid of B. umbellata, Wall., a Himalayan *Berberis*.

REFERENCES

(1) Buchner, *Ann.*, 24 (1835), 228; Polex, *Arch. Pharm.*, 6, II (1836), 265; Wacker, *Vjschr. prkt. Pharm.*, 10 (1861), 545; Hesse, *Ber.*, 19 (1886), 3190; Schulz, JOUR. A. PH. A., 15 (1926), 33; Späth and Kolbe, *Ber.*, 58 (1925), 2280; Späth and Polgar, *Monatsh.*, 52 (1929), 117; Schulz and von Bruchhausen, *Arch. Pharm.*, 267 (1929), 617.

(2) Neppach, *Amer. J. Pharm.*, (1878), 373.

(3) Schilbach, see Wehmer, "Die Pflanzenstoffe," 1929.

(4) Parsons, *Pharm. J.*, 13, III (1882–1883), 46; Stübbe and Rüdel, *Arch. Pharm.*, 229 (1891), 631.

(5) Arata, *Rep. Pharm.* (1892), 45.

(6) Perkin, A. G., *J. Chem. Soc.*, 71 (1897), 1194.

(7) Chopra, Ghosh and Ratnagiriswaran, *Indian J. Med. Research*, 16 (1929), 776.

(8) Kondo and Tomita, *Arch. Pharm.*, 268 (1930), 549.

(9) Cromwell, *Biochem. J.*, 27 (1933), 860.

(10) Orekhov, *Arch. Pharm.*, 271 (1933), 323.

(11) Gurguel, Costa, da Silva, *Bull. assoc. Brasil. Pharm.*, 15 (1934), 11.

(12) Chatterjee, R., *J. Indian Chem. Soc.*, 17 (1940), 289.

(13) Chopra, R. N., "Indigenous Drugs of India," 1933, page 291.

(14) Hooker, J., "Flora of British India," Vol. I, page 110.

The Stabilization of Liquid Preparations Containing Pepsin*

By C. J. Klemme† and C. L. Boswell‡

INTRODUCTORY

The present work is a continuation of the study of liquid preparations containing pepsin for the purpose of stabilizing the activity of peptic systems. Heretofore, as mentioned by Klemme and Worrell (1), stability studies of pepsin preparations have been inconclusive, due primarily to the lack of a satisfactory method of assay. Klemme and Worrell had this objective in mind, namely, to develop a procedure which could be employed in the accurate assay of pepsin preparations for activity. In the experiments conducted in this research their method of assay has been used entirely.

In the past few years several enzymes have been isolated in pure crystalline form and their chemical nature studied. Pepsin, the enzyme with which we are primarily con-

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cerned, possesses the physical and chemical properties of an albumin, a typical primary protein, and its unusual proteolytic activity is generally associated with the particular arrangement of the constituent amino acids in the pepsin molecule (Northrop (2, 3)). Northrop (4) indicates that a major source of inactivation in the case of pepsin is due to denaturation of the enzyme protein.

Many factors, such as hydrogen ion concentration, temperature, purity of the enzyme, concentration of the enzyme, presence of added electrolytes and other substances, as well as time of exposure to these conditions, affect the activity of the particular enzyme in solution.

In attacking the problem of stabilizing the activity of liquid preparations containing pepsin, it was deemed fundamental that a study first be made of the influence of individual factors or variables on the maintenance of peptic activity over an extended period of time. The factors which have received special consideration include temperature, p_H, antioxidants (maleic acid, hydroquinone, resorcinol), preservatives (glycerol, alcohol, hexylresorcinol, "Merthiolate"), protective agents (acacia), low concentrations of amino acids (tyrosine), agitation and storage under nitrogen.

A number of preparations were made at suitable intervals, the initial ones having a single variable factor, whereas later ones possessed combinations of agents that seemed desirable as a result of a study of the stability of previous samples. All of the solutions were assayed at regular intervals throughout the duration of the study.

EXPERIMENTAL

Armour's U. S. P. Pepsin was used in making all of the preparations for stability tests, and Merck's Casein (according to Hammarsten) was employed as the substrate for determining the peptic activity.

In general, the preparations were made by dissolving the pepsin in distilled water (or a buffer solution prepared from 0.5*M* potassium acid phthalate and 0.5*M* sodium hydroxide), filtering the solution by suction, and then, after washing the filter with a small amount of distilled water, adding the other constituents of the preparation along with enough distilled water or buffer solution to bring the product up to the required volume.

Table I contains a selected list of the preparations studied. Only the initial and final assay figures are

Table I.—A Summary of Assay Results of Pepsin Solutions on Stability Tests

No. Prepn.	Variable Factors	Gm. Pepsin per 100 Cc. Initial	Gm. Pepsin per 100 Cc. Final	Per Cent Loss in Activity
5	"Merthiolate," 1:5000 p_H 4.5	4.9	5.1/12 mo.	0
27	"Merthiolate," 1:10,000 Under N_2 at 5° C. p_H 4.5	5.1	4.4/18 mo.	14
36	"Merthiolate," 1:10,000 Buffered, p_H 4.0	4.1	3.4/12 mo.	17
6	"Merthiolate," 1:10,000 Buffered, p_H 4.5	5.2	4.5/12 mo.	13
37	"Merthiolate," 1:10,000 Buffered, p_H 4.8	4.0	3.9/12 mo.	3
38	"Merthiolate," 1:10,000 Buffered, p_H 5.2	3.6	3.5/12 mo.	3
39	"Merthiolate," 1:10,000 Buffered, p_H 6.0	3.1	2.8/12 mo.	10
16	Acacia, 1% "Merthiolate," 1:2000 Under N_2 p_H 4.5	4.5	3.1/24 mo.	31
19	Tyrosine, 0.1% "Merthiolate," 1:2000 Under N_2 p_H 4.5	5.3	4.1/24 mo.	23
26	Alcohol, 12% "Merthiolate," 1:10,000 Buffered, p_H 4.5	5.5	4.8/12 mo.	13
8	Alcohol, 8% Under N_2 p_H 4.5	5.2	2.9/24 mo.	46
9	Alcohol, 12% p_H 4.5	4.7	4.5/12 mo.	4
13	Malic Acid, 1% p_H 2.8	5.2	0/8 mo.	100
31	Resorcinol, 1% Alcohol, 4% p_H 4.5	5.0	3.2/18 mo.	36
33	Hexylresorcinol, 1% Alcohol, 4% p_H 4.5	5.0	4.8/18 mo.	4
35	Magnesium Chloride, 0.5% Catechol, 1% Under N_2 p_H 4.5	3.5	2.1/12 mo.	40
22	Glycerol, 20% "Merthiolate," 1:5000 Buffered, p_H 5.0	4.8	4.7/12 mo.	2
10	Glycerol, 50% p_H 4.5	4.7	5.0/18 mo.	0
11	Glycerol, 90% p_H 4.5	4.7	4.8/18 mo.	0
40	Glycerite of Pepsin, N. F. VI p_H 4.2	9.9	10.2/12 mo.	0
42	Glycerol, 50% Aromatic Elixir, 50% p_H 4.4	5.0	4.8/18 mo.	4
44	Elixir of Pepsin, N. F. VI p_H 4.1	3.5	3.6/12 mo.	0
46	Compound Elixir of Pepsin, N. F. VI p_H 4.3	3.2	2.3/12 mo.	28
47	Elixir of Pepsin and Rennin, N. F. VI p_H 4.2	2.4	2.1/12 mo.	13
50[a]	"Aqueous Elixir of Pepsin" p_H 4.1	3.3	3.2/12 mo.	3

[a] Differs from the N. F. VI Elixir of Pepsin in that Aqueous Elixir is substituted for Aromatic Elixir, and that "Merthiolate," 1:5000, is added.

reported in this table. The constituents, other than pepsin, are listed under "Variable Factors." Most of the preparations were stored at room temperature in a closed container. Storage under an atmosphere of nitrogen or at refrigeration temperature was considered to represent a variable factor. Since the hydrogen ion concentration has proved to be of utmost importance, the approximate p_H of each preparation is included.

The limit of error in assaying a preparation from time to time is approximately 6%. If we assume that any preparation retaining 90% or more of the original activity after a year's time is stable for all practical purposes, then it is evident that several of those studied are promising from the standpoint of stability.

Undoubtedly the most important factor pertaining to the stabilization of peptic systems is the hydrogen ion concentration. Our results show that the optimum p_H range for storage of pepsin solutions is from p_H 4.5 to 5.5. A p_H below 4 is definitely unfavorable for the retention of proteolytic activity.

Although solutions containing "Merthiolate" as a preservative retained their activity quite well, a slight reduction of the mercury in the "Merthiolate" was noticeable after a few months of aging.

As a group, all of the preparations containing 20% or more of glycerol retained their original activity throughout the assay period.

Of the N. V. VI preparations, the Elixir of Pepsin and the Glycerite of Pepsin are the most stable. Synder, Goldberg and Gathercoal (5) conclude that the greater stability of the N. F. VI Elixir of Pepsin, as compared to the N. F. V Elixir, results from a better control of the hydrogen ion concentration. The N. F. VI Elixir is buffered at p_H 4.1 approximately, whereas the p_H of the N. F. V product is 2.5. Preparation 13, having a p_H of 2.8, was completely inactive at the end of eight months.

Experiments on the effect of shaking pepsin solutions were also performed. Klemme and Boswell (6) reported that vigorous mechanical agitation for periods of six hours or less produced no discernible losses in peptic activity, whereas sizable losses in proteolytic power occurred after vigorous shaking for over twelve hours. Further experiments in this connection indicated that certain preparations were stable to mild mechanical agitation for a period of one week. Before assaying the preparations following agitation, the froth was allowed to settle completely. Consequently, the statements in the National Formulary regarding the reduction in proteolytic activity of pepsin solutions by excessive shaking or stirring are unnecessary.

A pepsin solution is essentially a colloidal dispersion of a protein, and its stability is determined primarily by the hydrogen ion concentration of the solution and the water of hydration surrounding the pepsin molecules. The stabilizing action of glycerol may be due to its adsorption on the pepsin molecules, forming a film which has a strong attraction for water molecules.

From a careful study of the pepsin solutions on stability tests, we have formulated four preparations which will undergo no noticeable color change or sedimentation, and which will retain their peptic activity over a long period of time without suffering any appreciable loss in activity.

The suggested formulas are as follows:

FORMULA I

Pepsin, U. S. P.	50 Gm.
Citric Acid Crystals	10 Gm.
Exsiccated Sodium Phosphate	14.5 Gm.
Glycerol	200.0 cc.
Aromatic Elixir	200.0 cc.
Distilled Water, a sufficient quantity, To make	1000 cc.

FORMULA II

Pepsin, U. S. P.	35 Gm.
Citric Acid Crystals	10 Gm.
Exsiccated Sodium Phosphate	14.5 Gm.
Distilled Water	300.0 cc.
Glycerol	200.0 cc.
Aromatic Elixir, a sufficient quantity, To make	1000 cc.

Note: The p_H of the above preparation is approximately 5, whereas the N. F. VI Elixir of Pepsin has a p_H slightly over 4.

FORMULA III

Pepsin, U. S. P.	100 Gm.
Glycerol	500 cc.
Distilled Water, a sufficient quantity, To make	1000 cc.

Note: The above product differs from the N. F. VI Glycerite of Pepsin in that the diluted hydrochloric acid is omitted. The p_H of the above product is approximately 4.5.

FORMULA IV

Pepsin, U. S. P.	50 Gm.
Citric Acid Crystals	10 Gm.
Exsiccated Sodium Phosphate	14.5 Gm.
Glycerol	500.0 cc.
Distilled Water, a sufficient quantity, To make	1000 cc.

The method of preparation of the above products is of considerable importance. The suggested procedure is as follows: Dissolve the citric acid and exsiccated sodium phosphate in a sufficient amount of distilled water (about 200 to 300 cc.), add the pepsin and stir until dissolved. (In Formula III, the buffer substances are omitted.) Allow the solution to stand over night in a refrigerator. Filter the solution, preferably by suction, and wash the filter thoroughly with distilled water. To the clear filtrate add the glycerol and the remaining constituents along with enough distilled water to bring the product up to the required volume. Mix thoroughly and place in a closed container.

COMMENTS

In making the above preparations it is not necessary to avoid agitation. The formulas may be varied slightly as long as the p_H is maintained in the neighborhood of 4.5 to 5.5, and as long as 20% or more of glycerol is present. An alcoholic concentration above 15% should be avoided. For best results, the method of preparation should be carried out as directed above. If filtration by suction is not available, filtration by gravity may be employed, being certain that the filter paper is washed thoroughly with distilled water. Finally, the preparations should be assayed for peptic activity before placing in permanent flasks.

BIBLIOGRAPHY

(1) Klemme, C. J., and Worrell, L. F., JOUR. A. PH. A., 29 (1940), 263.
(2) Northrop, J. H., *J. Gen. Physiol.*, 13 (1930), 739.
(3) Northrop, J. H., *Ibid.*, 14, (1931), 713.
(4) Northrop, J. H., *Ibid.*, 16 (1932), 33.
(5) Snyder, R. K., Goldberg, L., and Gathercoal, E. N., JOUR. A. PH. A., 26 (1937), 541.
(6) Klemme, C. J., and Boswell, C. L., *Ibid.*, 29 (1940), 497.

Carcinogenics and Medicine*

By Seward E. Owen†

This report deals with a consideration of the carcinogenic chemicals, their sources, biological activity, chemistry and methods of detection as they relate to medicine.

Certain tars were first shown to be capable of producing cancer in experimental animals by Yamagiwa and Ichikawa (1) in 1913. Since that time much progress has been made in the clarification of the chemical formulas of substances capable of inducing malignant changes in the tissues of experimental animals. Important contributions are those of Bloch and Dreifuss (2) who showed that carcinogenic material in tar was contained in the nitrogen-free, neutral, high-boiling fraction. That the active substances were hydrocarbons was indicated by the work of Kennaway (3). Mayneord (4) first applied the fluorescent spectrum method to the problem. Heiger (5) later noted that many carcinogenics displayed similar fluorescence spectra. The absorption spectrum of 1:2:5:6 dibenzanthracene was first studied by Clar (6) and later by Chalmers (7). These early spectrum studies led the English scientists to try many compounds with similar spectra for possible carcinogenic effects on animals, with the result that 1:2:5:6 dibenzanthracene was found to be a potent cancer-causing agent when applied in solution to the skin of animals. By this technique cancers of the squamous variety were induced. Later Burrows, *et al.* (8), noted that the same agent in contact with connective tissue produced tumors of this tissue. Cook, *et al.* (9), synthesized a large number of similar compounds which were tested biologically. Among these were methylcholanthrene and 1:2 benzpyrene, both highly carcinogenic.

The most important carcinogenic chemicals that may exist in coal tar are 1:2 benzpyrene, 1:2:5:6 dibenzanthracene and possibly methyl cholanthrene. These are now made synthetically for experimental use and may be obtained from the standard chemical houses. Reviews from the chemical standpoint of carcinogenics have been presented by Cook and Kennaway (10) and by Fieser (11). These are rather exhaustive and complete. Many derivatives of the above carcinogens as well as some newer compounds are mentioned in the above papers. Other reviews consider the carcinogenics, sex hormones and sterols, all of which have the phenanthrene nucleus Owen (12) and Dodds (13). In this connection it is of interest to point out that some synthetic carcinogenics are estrogenic and that rather potent estrogenics may be derived from certain closely related, chemically, carcinogenic agents.

The test methods to demonstrate carcinogenic activity are those adopted by the English workers which consist of applying the hydrocarbons at 0.3% in benzene solution to the interscapular region of mice

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