sion line:

$$\log LT_{50} = 1.6987 - 0.6493 \log R \qquad \text{(Eq. 38)}$$

A Student's t test indicated the two slopes were not significantly different ($t = 0.14$, $p > 0.25$), hence the data for all twelve compounds were pooled and these yielded the regression line:

$$\log LT_{50} = 1.6755 - 0.6276 \log R \qquad \text{(Eq. 39)}$$

The correlation coefficient was -0.848 ($p < 0.001$). The plot of these data with the line corresponding to Eq. 39 drawn through the points is shown in Fig. 7. If one assumes that R is a reflection of Ci the relevance these data have to the theory discussed and to the goldfish problem is evident.

The relationship between the theoretical equations derived in this report and the equation relating intensity of pharmacologic response to drug concentration reported by Wagner (15) in the first paper of this series will be discussed in a future publication.

REFERENCES

(1) W. Ostwald, *Arch. Ges. Physiol.*, **120,** 19(1907).

(2) E. B. Powers, *Illinois Biol. Monographs*, **4,** 127(1917); (Powers reviews all earlier literature).

(3) N. L. Drake and R. L. Bushey, *J. Am. Chem. Soc.*, **54,** 2930(1932).

(4) G. Levy and S. P. Gucinski, *J. Pharm. Exptl. Therap.*, **146,** 80(1964).

(5) G. Levy and K. E. Miller, *J. Pharm. Sci.*, **53,** 1301(1964).

(6) *Ibid.*, **54,** 1319(1965).

(7) N. A. Hall and W. L. Hayton, *ibid.*, **56,** 304(1967).

(8) M. Gibaldi and C. H. Nightingale, *J. Pharm. Sci.*, **57,** 226 (1968).

(9) *Ibid.*, **57,** 1354(1968).

(10) A. J. Clark, *J. Physiol.*, **61,** 530(1926).

(11) "Molecular Pharmacology. The Mode of Action of Biologically Active Compounds," vol. I, E. J. Ariens, Ed., Academic Press, New York, N. Y., 1964, p. 146.

(12) W. D. M. Paton and D. R. Waud, *Arch. Exptl. Pathol. Pharmakol.*, **248,** 124(1964).

(13) W. Morozowich, T. Chulski, W. E. Hamlin, P. M. Jones, J. I. Northam, A. Purmolis and J. G. Wagner, *J. Pharm. Sci.*, **51,** 993(1962).

(14) O. L. Davies, "Design and Analysis of Industrial Experiments," Hafner, New York, N. Y., 1954, p. 287.

(15) J. G. Wagner, *J. Theoret. Biol.*, **20,** 173(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 31, 1969, from the *College of Pharmacy, The University of Michigan and the College of Pharmacy and Pharmacy Service, University Hospital, The University of Michigan, Ann Arbor, MI 48104*

Accepted for publication May 28, 1969.

This work was supported in part by General Research support, FR 5571-05, U.S. Public Health Service, Bethesda, Md. and in part by the National Science Foundation Graduate Traineeship Program.

Effects of Some Enzymes, Surface-Active Agents, and Calcium Chloride on the Aqueous Extraction of Alkaloids from Belladonna Leaves

JOSÉ HELMAN

Abstract □ Treatment of belladonna leaves with the enzymes described, prior to extraction with surface-active agents and calcium chloride, results in higher yields. This was observed in simple aqueous extraction and also when hydrochloric acid was added.

Keyphrases □ Alkaloid extraction—belladonna leaves □ Surfactant effect—alkaloid extraction □ Enzymes effect—alkaloid extraction □ Calcium chloride effect—alkaloid extraction

Previous studies concerning the effect of various surface-active agents on the extraction of alkaloids have shown that the yield in aqueous medium varies according to the agent used. In general the yield decreases with anionic agents, is slightly increased with nonionic, and much more so with cationic agents. Nonionic agents such as polyoxyethylene sorbitan monolaurate and mono-oleate, sorbitan laurate ester, polyethylene glycols 400 and 600, propylene glycol, and glycerol esters (1–3) have been assayed in the extraction from hyoscyamus, belladonna, ipecac, cinchona, hydrastis, *etc.*

Experiments performed by Cadórniga *et al.* (4, 5) with anionic agents proved that at low concentrations the yield decreases, but increases at high concentrations. Results considerably above controls were obtained with cationic agents, especially with quaternary ammonium compounds (6, 7).

Gupta and Sen Gupta treated powdered kurchi (*Holarrhena antidysenterica*), belladonna, nux vomica, and ipecac with diastase prior to extraction (8). White *et al.* (9), in a study directed to obtain proteins and other kinds of cellular material from leaves, subjected these to the action of *Clostridium roseum* cultures, exposing them to an anaerobic fermentation.

The purpose of this work is to determine the yield of alkaloid extraction from belladonna, using aqueous media and with the aid of enzymes, surface-active agents, and calcium chloride.

EXPERIMENTAL

Materials—Powdered belladonna leaves (*Atropa belladonna*), 40 mesh (0.19 mm. sieve opening), dried at 60° were used.

Table I—Alkaloid Yield Obtained by Pretreatment of Belladonna Leaves with Enzymes in Extraction with Aqueous Dispersions of Surface-Active Agents and Calcium Chloride, at 30°C.

Agents Used	Water — Test No.	Water — Yield,[a] %	HCl, 2% — Test No.	HCl, 2% — Yield, %
Without Enzymes				
—	1	38.2	6	75.0
PSMO	2	40.4	7	75.7
DNaSS	3	16.5	8	76.7
CTAB	4	50.5	9	83.7
$CaCl_2$	5	25.3	10	32.7
With Malt Amylases				
—	11	44.0	16	87.0
PSMO	12	50.0	17	85.8
DNaSS	13	23.7	18	83.3
CTAB	14	56.2	19	87.0
$CaCl_2$	15	34.6	20	88.3
With Fungic Amylase				
—	21	40.0	26	87.5
PSMO	22	36.5	27	75.0
DNaSS	23	23.0	28	88.3
CTAB	24	79.5	29	102.0
$CaCl_2$	25	92.0	30	100.0
With Cellulose				
—	31	45.0	36	104.0
PSMO	32	60.0	37	111.0
DNaSS	33	31.3	38	93.4
CTAB	34	75.3	39	110.0
$CaCl_2$	35	88.3	40	104.0
With Protease				
—	41	60.0	46	96.0
PSMO	42	75.3	47	100.0
DNaSS	43	16.6	48	80.9
CTAB	44	83.7	49	112.0
$CaCl_2$	45	48.2	50	88.5
With Pectinase				
—	51	65.0	56	88.4
PSMO	52	65.4	57	90.0
DNaSS	53	16.5	58	85.4
CTAB	54	50.5	59	81.5
$CaCl_2$	55	105.0	60	110.0

[a] Results are expressed as a percentage of the analytical data obtained from evaluation of the powder.

Alkaloid content was estimated according to the Argentine Pharmacopoeia IV[1] (AP), except for a slight modification in which the final chloroformic extract was treated with anhydrous sodium sulfate to prevent contamination by the alkalinized aqueous medium with consequent distortion of results. The slight decrease in volume resulting from chloroform retention by sodium sulfate was taken into account when making the calculation. As suggested by Ridolfo and Guth (10), a mixture of chloroform and 2 ml. alcohol replaced the third addition of chloroform itself to promote a better elimination of the volatile bases.

Polyoxyethylene sorbitan mono-oleate USP (PSMO), dioctyl sodium sulfosuccinate NF (DNaSS), and cetyltrimethyl ammonium bromide[2] (CTAB) were used as nonionic, anionic, and cationic surface-active agents, respectively. Calcium chloride and all other reagents were according to AP specifications. Surface-active agents were used at concentrations above the CMC, *i.e.*, PSMO and DNaSS at 0.25% and CTAB 1%. Calcium chloride was used at a concentration of 1%.

The following enzymes were used:

Maltine or diastase (amylolytic malt enzyme), tested according to the French Pharmacopoeia (1937).

Amylolytic enzyme of fungic origin,[3] estimated by a procedure suggested by its manufacturers, based on a change of color in the enzyme-treated starch on addition of a diluted iodine solution. This enzyme acts within a pH range of 4.0–8.5. At room temperature 1 g. dextrinizes 1910 g. of starch in 1 hr., though faster at body temperature.

Cellulolytic enzyme[4] (obtained from a selected strain of *Aspergillus niger*) is usually contaminated to a small extent with other enzymes. The activity was calculated from a decrease in viscosity of a sodium carboxymethylcellulose dispersion. At 37° it acts in a pH range from 3.0–7.0, and even at extreme pH values 50% of its activity is retained.

Proteolytic enzyme[5] (isolated from *Carica papaya*) was tested according to AP procedure for papain. This enzyme acts within a pH range of 3.0–10.5.

Pectinolytic enzyme[6] was estimated by degradation of a quince jelly whose transmittance was read in a spectrophotometer.

Extraction Procedure—To a 250-ml. flask containing a 11.25-g. fraction of belladonna, 40 ml. of distilled water was added. The mixture was shaken mechanically for 10 min. and was held at 30° for 14 hr., after which the surface-active agent or salt, dispersed in 35 ml. distilled water, was added. After 20 min. stirring, the flask was kept for 10 hr. at the same temperature. At 2-hr. intervals for the first 6 hr. and hourly for the last 4 hr., the mixture was subjected to a 10 sec. stirring by hand. Then it was thoroughly mixed and finally filtered. Determinations of pH and surface tension were made and a fraction was used for alkaloid estimation, as previously stated. Another fraction was used to determine the dry extract through evaporation and drying at 100° to constant weight.

In another series of tests, 1.5 ml. of the initial 40 ml. of distilled water was replaced by 1.5 ml. of hydrochloric acid.

In the assays with enzymes, the following amounts were added with the initial 40 ml. distilled water: 0.225 g. maltine, 0.15 g. amylolytic enzyme, 0.075 g. cellulolytic enzyme, 0.075 g. proteolytic enzyme, and 0.225 g. pectinolytic enzyme. Similar tests were per-

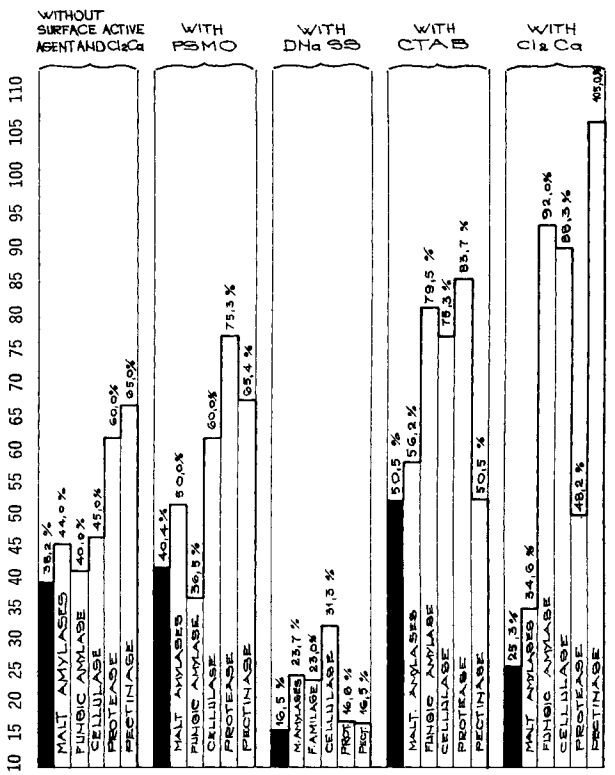

Figure 1—*Effect of enzymes on the extraction of belladonna with aqueous dispersions of calcium chloride and surface-active agents. Black columns correspond to tests without enzymes.*

[1] The USP procedure involves two chloroform evaporations while the Argentine Pharmacopoeia recommends three.

[2] Cetavlon, Imperial Chemical Ind. Ltd., London, England.

[3] Mylase 100, Wallerstein Co., New York, N. Y.

[4] Cellase 1000, Wallerstein Co., New York, N. Y.

[5] Prolase 300, Wallerstein Co., New York, N. Y.

[6] Klerzyme 200, Wallerstein Co., New York, N. Y.

Table II—Surface Tension, pH and Dry Extract of Extracts from Table I are Shown with Corresponding Index of Selectivity[a] of Each Solvent

Agents Used	Water					HCl, 2%				
	No.	Surface Tension	pH	Dry Extract	Selectivity Index	No.	Surface Tension	pH	Dry Extract	Selectivity Index
Without Enzymes										
—	1	55.0584	5.4	18.7	0.02	6	49.1819	2.3	27.3	0.02
PSMO	2	48.8929	5.5	20.7	0.02	7	49.1819	2.4	27.3	0.02
DNaSS	3	54.9504	5.5	19.8	0.01	8	49.1189	2.4	26.7	0.03
CTAB	4	44.0470	5.2	20.3	0.03	9	30.1798	2.3	28.7	0.03
$CaCl_2$	5	55.2755	5.1	25.5	0.01	10	37.0671	2.3	26.7	0.01
With Malt Amylases										
—	11	54.9504	4.9	19.3	0.01	16	50.9130	2.4	30.0	0.03
PSMO	12	47.1003	4.4	20.0	0.01	17	45.7452	2.5	29.1	0.03
DNaSS	—					18	45.7004	2.6	29.4	0.02
CTAB	14	44.0470	5.2	25.3	0.02	19	30.1491	2.6	35.3	0.03
$CaCl_2$	15	55.2170	4.7	29.0	0.01	20	51.2737	2.4	38.0	0.02
With Fungic Amylase										
—	21	55.0584	5.2	21.0	0.02	26	51.1236	2.6	28.8	0.04
PSMO	22	50.9130	4.9	24.0	0.01	27	49.2300	2.6	29.3	0.02
DNaSS	23	50.8735	5.3	21.3	0.01	28	49.2300	2.6	29.4	0.03
CTAB	24	44.1337	4.5	23.0	0.04	29	30.2389	2.7	34.7	0.05
$CaCl_2$	25	55.3829	5.1	28.7	0.04	30	39.2857	2.6	33.9	0.03
With Cellulase										
—	31	55.1671	5.1	18.9	0.01	36	51.1736	2.5	30.7	0.02
PSMO	32	50.9735	5.1	23.3	0.03	37	44.3938	2.6	31.4	0.03
DNaSS	33	50.9735	5.1	20.7	0.01	38	47.6615	2.5	27.0	0.03
CTAB	34	44.1337	5.0	22.3	0.04	39	30.2094	2.3	26.1	0.06
$CaCl_2$	35	55.4361	4.8	26.4	0.04	40	44.5239	2.4	32.5	0.03
With Protease										
—	41	50.9130	4.5	20.3	0.02	46	49.3410	3.3	29.1	0.03
PSMO	42	50.9130	5.1	20.1	0.02	47	49.2301	2.6	28.2	0.02
DNaSS	43	55.2755	5.3	20.7	0.01	48	49.1189	2.3	27.8	0.03
CTAB	44	45.7452	5.2	23.2	0.05	49	30.2691	2.4	35.4	0.03
$CaCl_2$	45	54.5855	5.1	29.7	0.02	50	44.4805	2.4	32.7	0.03
With Pectinase										
—	51	50.7734	5.7	23.7	0.03	56	55.4361	2.7	37.0	0.02
PSMO	52	50.7734	4.9	19.0	0.04	57	55.3301	2.7	32.0	0.02
DNaSS	53	55.0045	5.4	16.1	0.01	58	45.8349	2.7	30.0	0.02
CTAB	54	47.2861	5.1	12.0	0.08	59	30.2094	2.7	39.1	0.02
$CaCl_2$	55	55.2250	4.4	26.4	0.08	—				

[a] The selectivity index (denomination used by other authors) is expressed as a relation between the weight of alkaloids and that of the extract, from which the proportional weight of substances added to solvents was subtracted. Both values refer to 100 g. of drug.

formed except that 1.5 ml. of the 35 ml. distilled water added after the 14 hr. period of heating at 30° was replaced by 1.5 ml. hydrochloric acid. Thus the chance of surface-active agents, salt or acid interfering with enzymatic action was eliminated.

Since decoction of belladonna leaves produces a significant decrease in the yield, assays were also performed to determine the effect of enzymes under such conditions. Enzymes were therefore added both before and after decoction.

To ascertain whether surface-active agents or enzymes could by themselves modify the results of alkaloid estimation, assays were carried out using them at the same concentrations as previously, but without belladonna. The values found did not distort results, except in the case of CTAB, which was considered on making the calculations.

The reported results represent the arithmetic mean of at least two runs for each experiment. In no case did deviation exceed 5%.

Surface Tension—For this purpose only information concerning the various extracting liquids was found necessary. Surface tension was expressed as a relationship between drop number and density of the extracting liquids and those values obtained for water under similar conditions, multiplied by surface tension of water.

RESULTS AND DISCUSSION

Table I shows that surface-active agents dissolved in water follow the order of efficiency previously stated. The addition of hydrochloric acid to a concentration of 2%, thus lowering the pH of the extracts from about 5.0 to 2.5, enhanced DNaSS yield. The order of efficiency was then: CTAB > DNaSS > PSMO > HCl > $CaCl_2$, showing an increase of 20–80%, or more, over the yields obtained with simple aqueous extraction (Figs. 1 and 2).

The slight increase observed with the use of PSMO might be ascribed to PSMO effect on the permeability of membranes rather than to a solubilizing effect on alkaloids, in this case lacking. It might also be attributed to dissolution of: (*a*) certain membrane compounds; (*b*) cellular components to which alkaloids would be bound; or (*c*) complexes containing alkaloids (2, 11).

Brochmann-Hanssen's hypothesis on CTAB mechanisms of action (7) to explain the increase in yields observed, does not exclude the existence of other mechanisms, which might act simultaneously, related to the colloidal nature of this surface-active agent.

DNaSS gave lower yields than controls, except in Assays 8 and 28. It should be noted that DNaSS at 0.25% concentration precipitates when mixed with equal amounts of a 1% solution of atropine sulfate, but not with a 0.05% solution.

Admittedly the low yields found with anionic surface-active agents are largely due to their ability to precipitate alkaloids. But at low pH values, cinchona alkaloids do not precipitate either with DNaSS or sodium lauryl sulfate (12). The latter never precipitates with atropine sulfate, even at pH 6.5. Yet the yields obtained are lower than those found when no surface-active agents were used. An explanation might be that the larger micelles which constitute the complexes of anionic surface-active agents and alkaloids, would hinder, or even prevent them from penetrating cell membranes and the tissue itself. A mechanical factor may also be involved if large micelles (without alkaloid content) obstruct the diffusion

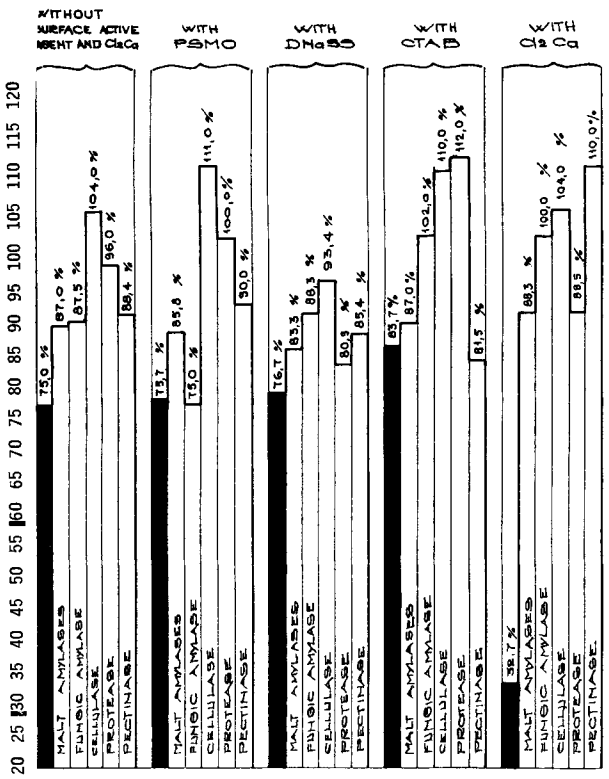

Figure 2—*Effect of enzymes on the extraction of belladonna with acid dispersions of calcium chloride and surface-active agents. Black columns correspond to tests without enzymes.*

channels. The micellar size of these complexes is under investigation in the laboratory.

In general, pretreatment with enzymes promoted higher yields. Experiments carried out with malt amylases gave increased yields as compared to those obtained without using enzymes, especially in acid extraction (Tests 11 to 20, and 1 to 10). Different results were obtained with amylolytic enzyme of fungic origin. Tests 24 and 29 with CTAB showed a higher increase, while Tests 25 and 30, with $CaCl_2$, gave yields close to 100%. The other enzymes tested also showed enhancing effects, yielding in most cases about 90% and, in some, exceeding 100%. CTAB was found to be the most effective agent in both aqueous and acid extraction, reaching its highest values after treatment with fungic amylase, cellulolytic, and proteolytic enzymes. After treatment with these enzymes, the yield in acid extraction exceeded 100%. Similar results were obtained with $CaCl_2$ and pectinolytic enzyme (see Figs. 1 and 2).

Treatment with enzymes before and after a 5 min. decoction of belladonna leaves gave low yields, following this order of activity: pectinase > cellulase > fungic amylase > proteinase.

Table II shows that the pH of extracts obtained from nonacid aqueous extraction without using enzymes is, generally, higher than that of extracts obtained with enzymes. Variations in pH values depended upon enzymes used, and were less evident in acid extraction.

In general, aqueous extraction provided higher surface-tension values than acid extraction, while the latter gave higher yields of dry extracts. Again, these values varied according to the enzyme used.

REFERENCES

(1) G. P. Srivastava and T. N. Chadha, *J. Am. Pharm. Assoc.*, **52,** 299(1963).

(2) W. J. Butler and G. A. Wiese, *ibid.*, **42,** 382(1953).

(3) S. Derkatch, A. Goldberg, J. Stigi, I. Greenberg, and L. D. Fonda, *Drug Std.*, **23,** 32(1955).

(4) R. Cadórniga Carro, *Anal. Rev. Acad. Farm.*, **28,** 27(1962).

(5) R. Cadórniga Carro, *Farmaco Pavia, Ed. Prat.*, **17,** 646 (1962).

(6) T. A. Mosqueira, *Farmacognosia Madrid*, **18,** 167(1958); through *Chem. Abstr.*, 9571c(1959).

(7) E. Brochmann-Hanssen, *J. Am. Pharm. Assoc., Sci. Ed.*, **43,** 27(1954).

(8) H. N. Gupta and S. B. Sen Gupta, *J. Indian Chem. Soc. Ind. News Ed.*, **9,** 124(1946).

(9) J. W. White, Jr., L. Weil, J. Naghski, E. S. Della Monica, J. J. Willaman, *Ind. Eng. Chem.*, **40,** 293(1948).

(10) A. S. Ridolfo and E. P. Guth, *J. Am. Pharm. Assoc., Sci. Ed.*, **37,** 505(1948).

(11) R. Cadórniga Carro, E. Otero Aenlle, and M. de la C. Ares Posadas, *Prod. Pharm.*, **12,** 441(1957).

(12) J. Helman, Tesis doctoral, Universidad Nacional de Buenos Aires (1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 1, 1967 from the *Pharmaceutical School, National University of Córdoba, Córdoba, Argentina*

Accepted for publication June 5, 1969.

The author expresses his appreciation to Dr. Zenón Lugones for courteously reviewing the manuscript and to the auxiliary teaching staff for their collaboration in the experimental part.

Presented to the Pharmaceutical Section, VII Panamerican Biochemical and Pharmaceutical Congress, Buenos Aires, 1966.

Abstracted from a general study on yields in active materials alkaloids or otherwise, from vegetal substances after enzymati pretreatment and with the aid of surface-active agents and/or salts.