in a few animals and thus increases their oxygen consumption rather than decreases it. We have found no marked changes in respiratory rates beyond those produced by the tranquilizing effect of the barbiturate. The rats showed sedation and light anesthetic effects of the drug. They could be easily aroused to activity by mechanical stimulation and they retained a good degree of skeletal muscle tone at the dosage levels indicated in Table I. The averages of the rate of O_2-consumption per minute of the various groups of animals are shown separately in Table II. In 5 rats included in this series, where Seconal produced slight excitation, the average O_2-consumption rate increased from a normal of 4.4 cc. to 5.10 cc. per minute. There is a greater degree of depression of oxygen consumption in the female rats than in the males.

CONCLUSIONS

In conclusion, the results indicate that in the majority of rats a significant reduction in oxygen consumption (metabolic rate) occurs when subanesthetic doses of Sodium Seconal are injected subcutaneously. In this paper the mechanism of the depressant effects of Seconal on oxygen consumption is not investigated. It also appears that Seconal, in the doses used, did not produce any changes in the respiratory rate and that muscular relaxation was not a contributory factor in the reduction of oxygen consumption under our experimental conditions.

REFERENCES

(1) Guedel, Arthur E., *Anesthesia and Analgesia*, **15**, 152–57(1936).
(2) Anderson, H. H., Chen., M. Y., and Leake, C. D., *J. Pharmacol.*, **40**, 215 (1930).
(3) Lee, M. O., *Am. J. Physiol.*, **85**, 388(1928).
(4) Siebert, W. J., and Thurston, E. W., *Proc. Soc. Exptl. Biol. Med.*, **29**, 650(1931).
(5) Kleiber, M., and Saunders, F. J., *ibid.*, **36**, 377(1937).
(6) Peoples, S. A., *Anesthesia and Analgesia*, **17**, 130(1938).
(7) Deuel, H. S., Chambers, W. H., and Milhorat, A. T., *J. Biol. Chem.*, **66**, 249(1926).
(8) Boothby, W. M., and Rowntree, L. G., *J. Pharmacol.*, **22**, 99 (1923).
(9) Peoples, S. A., *Science*, **94**, 373 (1941).

The Lipolytic Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and EDWIN E. WILSON‡

A method of analysis which can be used successfully to determine the lipolytic activity of Pancreatin, U. S. P., is presented.

PANCREATIN U. S. P. contains the enzymes steapsin, amylopsin, and trypsin. The U. S. P. gives the minimum standards required for amylopsin and trypsin, but gives no minimum lipolytic activity. The only pharmacopœia requiring such standards is the British Pharmacopœia (4) which uses "the cream of fresh milk" as a substrate.

It appears that the reasons for not giving the lipase activity of Pancreatin, U. S. P. are the following:

(*a*) the inability to obtain a preparation with a large amount of lipase;
(*b*) the lack of a good assay; and
(*c*) the labile character of lipase.

The object of this investigation is to determine the lipolytic activity of Pancreatin U. S. P. and to find a method of assay whereby uniform results may be obtained. The degree of activity which is to be determined will not be based upon physiological conditions. This determination is impossible, since neither the length of time of the action of lipase in the animal body nor the influence of all the physiological activators or inhibitors has as yet been determined. For the same reasons minimum requirements given for trypsin and amylopsin in Pancreatin U. S. P. are not based upon the physiological action of these substances, and should not be so interpreted. It should be clearly understood that the degree of activity is merely determined under specific conditions suitable for analytical purposes, which may have little relationship to physiological conditions.

The literature of the biochemical journals gives numerous methods for the assay of lipase. A number of the better-known

* Received Sept. 4, 1946, from the Massachusetts College of Pharmacy, Boston.
Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, 1946.
† Associate Professor of Chemistry, Massachusetts College of Pharmacy.
‡ This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Edwin E. Wilson, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

methods of determination were tried and abandoned. The assay formulated by Willstätter, Waldschmidt-Leitz, and Memmen (1), with certain modifications, was most satisfactory for this investigation of U. S. P. Pancreatic lipolytic activity.

EXPERIMENTAL ANALYSIS

The Lipolytic Activity of Pancreatin U. S. P.—The determination of pancreatic lipase, as formulated by Willstätter, Waldschmidt-Leitz, and Memmen (1), is performed by titrating the liberated fatty acids produced as a result of the action of the fat-hydrolyzing enzyme steapsin, upon the substrate, olive oil, after digestion at 30° for one hour. The digestion mixture contains the enzyme preparation, ammonia-ammonium chloride buffer, calcium chloride, albumin, and olive oil. This reaction mixture is titrated in an alcoholic medium with 0.1 *N* alcoholic potassium hydroxide, using thymolphthalein as the indicator. The amount of 0.1 *N* alkali used, corrected for control, is the amount required by the liberated fatty acids. Willstätter and co-workers then converted the amount of alkali used into per cent of fat split. The per cent of fat split was then translated into lipase units. One Lipase Unit is the amount of enzyme required under the conditions specified above to split 24% of 2.5 Gm. of olive oil of a saponification value of 185.

If the splitting is more than 24%, the experiment should be repeated with one-half as much of the enzyme as was used before. If the splitting is less than 10%, the experiment should be repeated with twice as much enzyme. The percentage must be not less than 10% nor more than 24%; otherwise the lipase units cannot be determined. These limits have been incorrectly reported in many standard textbooks (3).

The Accuracy of the Assay Method of Willstätter, Waldschmidt-Leitz, and Memmem.—The digestion mixture of Willstätter, composed of several water-insoluble substances, fails to give a reaction that is uniform for the entire period of digestion. The presence of a water-insoluble substrate would seem to militate against uniform digestion, and this assumption was confirmed experimentally. Upon repetition of the experiment, a series of divergent readings was obtained. The average titration value was 12.76 cc. The standard variation computed from these results was 1.12 cc. The presence of this sizable variation suggested the search for a satisfactory inert emulsifying agent which could be used to get more nearly uniform reactions and consequently more nearly comparable titration values.

The Value of Bentonite in the Analysis of U. S. P. Pancreatic Lipolytic Activity.—After a search for inert emulsifying agents which would give more nearly homogeneous reaction mixtures, bentonite was found to be the most satisfactory substance tried. The use of a blank determination ruled out any effects that bentonite might have upon the reagents which could possibly lead to an erroneous interpretation of enzymatic activity.

Three cubic centimeters of Magma of Bentonite N. F. was used in place of the 3 cc. of water used by Willstätter. The use of bentonite reduced the standard variation from 1.12 cc. to 0.27 cc.

The Amount of Pancreatin U. S. P. That Can Be Used to Determine Its Lipolytic Activity by the Willstätter Method in the Presence of Bentonite.—It was found that more than 400 mg. of Pancreatin U. S. P. in the presence of bentonite failed to give uniform results. This was also true in the absence of bentonite. Any amount between 200 and 400 mg. of Pancreatin U. S. P. analyzed gave results from which a satisfactory and uniform curve could be plotted.

The Effect of Bile Salts on the Titration Values in the Presence of Bentonite.—Bile salts produced a significant lipase activation when added to the digestion mixture at the start, if the medium was alkaline; but the yellow color imparted to the reaction mixture, because of the bile salts, made it very difficult to get a sharp end point. Since a sufficient degree of lipase activity is obtained without the use of bile salts, and since the end point is more readily determined in the absence of them, it was deemed advisable to determine U. S. P. lipase activity without making further use of this impure activator.

The Abandonment of Albumin as an Activator.—The influence of albumin on lipase activity is believed to be something more than that of activation. Willstätter used albumin to compensate for the inhibitory effect of certain unidentified substances found in pancreatic preparations. The use of albumin for this purpose is subject to criticism. In the first place, it is difficult to get two samples of albumin of identical composition. In the second place, Pancreatin U. S. P. contains a proteolytic enzyme which acts upon albumin to form acids which influence the titration value materially (2). Since the lipase activity is based upon the amount of alkali required to neutralize the acids produced as a result of lipolytic activity, every precaution must be taken to avoid the formation of acids in any other way.

The titration values of lipase activity were sufficiently high in the absence of albumin to warrant its elimination, and the acidity so obtained is a true index of the amount of fatty acids produced as a result of pancreatic lipolytic activity.

The standard variation for the titration values in the presence of albumin and bentonite was found to be 0.27 cc. In the absence of albumin but in the presence of bentonite it was found to be 0.19 cc.

The Effect of Various Amounts of Bentonite on Lipolytic Activity of Pancreatin U. S. P. in the Absence of Albumin.—It appears that from 3 to 5 cc. of Magma of Bentonite N. F. produces the best results for analysis in the absence of albumin, and that this amount of bentonite has no appreciable effect upon the enzyme. The chief value of the bentonite is the production of a uniform mixture, by

means of which more of the fat is brought into contact with the enzyme, and which consequently gives a higher titration value in a given period of time.

The Amounts of Pancreatin U. S. P. That Can Be Used to Determine Its Lipolytic Activity in the Absence of Albumin but in the Presence of Bentonite.—It was found that more than 400 milligrams of a sample of Pancreatin U. S. P. could not be used if the lipase content were to be determined by the Willstätter Method. It was thought to be advisable to determine the amount of Pancreatin U. S. P. that could be assayed by this method in the absence of albumin but in the presence of bentonite. Here, too, it was found that 400 mg. was the upper limit that could be used to make this determination.

A Comparison of the Different Methods Used to Determine the Lipolytic Activity of a Sample of Pancreatin U. S. P.—Bentonite favors the formation of a finely divided substrate, giving a higher degree of digestion and more nearly uniform results. It appears that albumin either activates the lipase, neutralizes the action of some inhibitory substance, or serves as a substrate for the proteolytic enzymes in pancreatin. The increase in acidity may be due to either one or all of these three actions of albumin. A comparison of the data obtained by different methods of assay is shown in Table I.

TABLE I.—A COMPARISON OF PANCREATIC LIPOLYTIC ACTIVITY OF DIFFERENT METHODS OF ASSAY

Method of Analysis Used	Cc. of 0.1 *N* Alcoholic Potassium Hydroxide
Willstätter	10.66
	10.91
	8.31
	20.81
Willstätter (with bentonite)	20.00
	20.00
	17.36
Willstätter (with bentonite but without albumin)	17.21
	17.84

The Influence of Ether on the Lipolytic Activity of Pancreatin U. S. P.—In a series of experiments, 10 Gm. of Pancreatin U. S. P. was shaken intermittently with 500 cc. of ether for twelve hours at room temperature. The suspension was filtered and both the filtrate and residue were assayed. The ether-soluble fraction showed no lipolytic activity.

The ether insoluble fraction showed the same degree of lipolytic activity as that of an equal amount which had not been treated with ether. These results indicate that pancreatic lipase is neither soluble nor is it destroyed to an appreciable extent by ether. This finding leads one to question the use of ether for the purpose of stopping lipolytic activity as is done in the Willstätter Assay. The data of these findings are shown in Table II, and contradict the literature on the subject (3), which states that ether stops the action of lipase.

In a series of digestions, carried out as in previous experiments, the digestion mixture was washed with

TABLE II.—THE LIPASE ACTIVITY OF THE ETHER INSOLUBLE FRACTION OF PANCREATIN U. S. P.

Sample Analyzed	Mg. of Pancreatin Used	Cc. of 0.1 *N* Potassium Hydroxide
Untreated sample	300	16.07
	300	16.07
Ether insoluble fraction	300	15.06
	300	14.35

alcohol into a titration flask containing 20 cc. of ether. The mixture was made up to a volume of about 125 cc. with alcohol, and was titrated with 0.1 *N* alcoholic potassium hydroxide. In the control flasks, numbers 4, 5, and 6, (Table III), the pancreatin was added just before the titration. Flasks 1, 2, and 3 were titrated one hour after adding the pancreatin and were then allowed to sit for forty-eight hours at room temperature and again titrated. At the end of forty-eight hours additional potassium hydroxide was required to neutralize the acid which had been formed during this time. Flasks 7 and 8 were not titrated until they had sat for forty-eight hours at room temperature.

From these titration values it may be seen that the ether does not destroy the lipase activity of Pancreatin U. S. P. If, on the contrary, ether destroyed the lipase action then the readings should have been no greater for flasks 7 and 8 than the total titration values given for numbers 1, 2, and 3. These data suggest that the retarding effect in the first three flasks were due to either the alcoholic potassium hydroxide or the *p*H of the solution. It is obvious from these findings that ether may retard, but it does not destroy, the lipolytic activity of Pancreatin U. S. P.

TABLE III.—THE EFFECT OF ETHER ON LIPOLYTIC ACTIVITY

Flask No.	Mg. of Pancreatin	Cc. of Alkali after 1 Hr. Digestion	Cc. of Alkali 48 Hr. after Adding Ether	Total Cc. of 0.1 *N* Potassium Hydroxide
1	300	54.6	3.2	57.8
2	300	55.0	5.1	60.1
3	300	54.3	3.2	57.5
4 (control)	300	33.4	1.2	34.6
5 (control)	300	33.9	1.5	35.4
6 (control)	300	34.1	1.5	35.6
7	300	..	84.1	84.1
8	300	..	82.5	82.5

The Lipolytic Activity of Pancreatin U. S. P. in the Absence of a Buffer, but in the Presence of Bentonite.—Since bentonite was found to be an efficient emulsifying agent whose action as a buffer has not been tested, it was felt that it might serve a double purpose and that the ammonia-ammonium chloride buffer might advantageously be eliminated. However, the titration values obtained in the absence of the ammonia-ammonium chloride buffer were too low for advantageous elimination.

An Improved Method of Analysis for the Determination of Pancreatic Lipase.—Into a glass-stoppered 50-cc. Erlenmeyer flask, introduce exactly 2.5 Gm. of Olive Oil U. S. P., 3 cc. of Magma of Bentonite N. F., 0.5 cc of 2% calcium chloride solution, and 2 cc. of buffer (0.66 cc. of normal ammonium hydroxide and 1.34 cc. of normal ammonium chloride). Rotate this mixture several times so as to produce a thick emulsion. Make the enzyme preparation containing 300 mg. of pancreatin, up to 10 cc. with distilled water; add this preparation to the flask and agitate vigorously for one minute so as to emulsify thoroughly the mixture. Place this reaction mixture in a constant temperature bath adjusted to 30°. Exactly one hour after the introduction of the enzyme preparation, remove the flasks and the controls, wash their contents with Alcohol U. S. P. into larger flasks containing 20 cc. of ether so as to make a total volume of 125 cc. Add twelve drops of 1% thymolphthalein and titrate immediately with 0.1 *N* alcoholic potassium hydroxide.

Do not add the enzyme preparation to the control flasks until they are removed and ready for titration. Subtract the titration of the control determination from the enzyme-digestion mixture in order to get the amount of alkali that is used to neutralize the fatty acids as a result of lipolytic activity.

The Lipase Activity of Commercial Samples of Pancreatin U. S. P.—To determine the lipase content of commercial samples of pancreatin, samples were obtained from different drugstores. These preparations were manufactured by three different pharmaceutical houses from whom was ascertained the exact age of each sample. These samples had been opened and stored under various conditions. Some samples were powdery, light-cream-colored preparations, while others were caked and were dark brown in appearance. These samples were assayed by the method given above. The analyses indicate that the age of the sample is not the factor most significant of its lipolytic activity. The data in Table IV show that some of the older samples contained almost as much lipase as did the more recent.

TABLE IV.—THE LIPASE ACTIVITY OF 300-MG. SAMPLES OF PANCREATIN COMING FROM VARIOUS MANUFACTURERS

Sample No.	Company	Age in Mo.	Cc. of 0.1 *N* Potassium Hydroxide
1	A	15	17.966
2	B	15	26.455
3	B	13	27.800
4	C	72	23.075
5	C	36	24.000
6	B	180	8.700
7	B	>180	1.050

The analyses seem to indicate that Pancreatin U. S. P. contains enough steapsin to be included in the minimum requirements of the official definition. An official method of assay similar to the one used for these assays could then be adopted.

RECOMMENDATION

The data obtained in this investigation suggest the possibility of introducing the following paragraph into the official definition of Pancreatin U. S. P. (5).

The potency of Pancreatin U. S. P. shall be such that when assayed as directed it shall convert not less than its own weight of Olive Oil U. S. P. into fatty acids in one hour.

The adoption of this paragraph would also require the introduction of an assay method, such as has been recorded in this thesis, into the official monograph of Pancreatin U. S. P.

SUMMARY

1. Pancreatin U. S. P. has a measurable amount of lipolytic activity according to Willstätter's method of analysis.

2. The results of the assay of Pancreatin will vary in accordance with its age and method of analysis, and probably with the method of manufacture and storage.

3. Bentonite is an inert emulsifying agent which is very useful in the determination of lipolytic activity.

4. Ether appears to have no effect upon lipolytic activity and may not be used to stop the reaction. However, the ether should be retained in the assay, because it dissolves the undigested olive oil and hence gives a clearer end point.

5. The color produced by bile salt preparations makes them undesirable activators in this determination.

6. Albumin should not be used in this assay since it is acted upon by the proteolytic enzymes present in the pancreatin.

7. A method of analysis has been worked out which can be used successfully to determine the lipolytic activity of Pancreatin U. S. P.

REFERENCES

(1) Willstätter, R., Waldschmidt-Leitz, E., and Memmen, F., *Z. physiol. Chem.*, **125**, 93(1923).
(2) Penau, H., and Guilbert, J., *J. pharm. chim.*, **25**, 9 (1937).
(3) Hawk, P. B., and Bergeim, O., "Practical Physiological Chemistry," ed. 11, P. Blakiston's Son and Company, Philadelphia, 1937.
(4) "British Pharmacopœia," Constable and Company, Ltd., London, 1932.
(5) "Pharmacopœia of the United States of America," Twelfth revision, Mack Printing Company, Easton, Pa., 1942.