14th. April, 1941.

APPLICATION FOR
GUNNING (VICTORIA JUBILEE) PRIZE (1941)
IN MATERIA MEDICA.

Submitted by
G.A. Levy, B.Sc. (Edinburgh, 1936), Ph.D. (Edinburgh, 1938),
Department of Materia Medica, Edinburgh University.

In the course of work for the degree of Ph.D., the candidate studied the derivatives of cocaine, and observations were made regarding the influence of substituent groups on the properties of the molecule as a whole.

[1] The local anaesthetic properties of certain heterocyclic compounds.


(a) The passage of inorganic substances from the blood to the tissues.

(b) The estimation of barbiturates in blood.

(c) Research carried out for the Ministry of Supply. This is governed by the Official Secrets Act, and a confidential report is therefore submitted separately.

The application is based on research carried out in the Department of Materia Medica under Professor A.J. Clark.
Material submitted for consideration.

(1) The local anaesthetic properties of certain heterocyclic compounds.

(2) Emergency preparation of pyrogen-free water.

(3) The distribution and clearance of drugs in the body.
   (a) The passage of iodides and iodates from the blood to the tissues.
   (b) The estimation of barbiturates in blood.
   (c) Research carried out for the Ministry of Supply. This is governed by the Official Secrets Act, and a confidential report is therefore submitted separately.

(1) The Local Anaesthetic Properties of Certain Heterocyclic Compounds.

In the course of work for the degree of Ph.D., the candidate studied the local anaesthetic properties of new pyrazoline derivatives. Three of these were found to have advantages over cocaine, and observations were made regarding the influence of substituent groups on the properties of the molecule as a whole. Reprint enclosed of paper in collaboration with Dr. H.B. Nisbet.

(2) Emergency Preparation of Pyrogen-Free Water.

On the outbreak of war, the candidate, in collaboration with Dr. J.C. Lees, undertook an investigation of the biological assay of fever-producing substances in infusion fluids, at the request of
the Department of Health for Scotland. An emergency method was worked out of freeing water from pyrogens by treatment with powdered charcoal. Reprint of paper enclosed.

(3) The Distribution and Clearance of Drugs in the Body.

Most of the research undertaken in the Department of Materia Medica by the candidate has been concerned with the development and application of chemical methods in the study of the distribution and clearance of drugs by the body, with a view to explaining certain aspects of the pharmacological actions of the substances concerned.

(a) The passage of iodides and iodates from the blood to the tissues.

As a basis for future work, the rate and volume of distribution of sodium iodide were determined. This drug was chosen for the first diffusion studies since it can readily be determined chemically, and because of its relatively simple manner of distribution. It is not fixed or altered by the tissues (i.e. significantly for present purposes), and is confined to the extracellular fluid. Some experiments were also carried out on the distribution of sodium iodate since it was thought that this might diffuse throughout the total body water as chlorates are claimed to do.

In these experiments, samples of blood drawn at intervals following intravenous injection of the drug into a cat were analysed for iodine. The results showed that distribution of iodide
throughout the body was complete in 60 minutes, the volume of
distribution at equilibrium between blood and tissues being 34% of
the body weight. With iodate, the volume of distribution at
equilibrium was about twice that for iodide, suggesting that it
entered regions impenetrable to the latter. In spite of this,
owing to an increased rate of distribution from the start, the
time required for equilibrium to be established between blood and
 tissues was about the same as for iodide, and there was no indic-
ation of the diffusion being a two stage process.

This work has not been published.

(b) The estimation of barbiturates in blood.

In a quantitative sense, members of the barbiturate group
can be obtained differing very widely in their actions, and it
was decided to investigate how far these differences could be
explained by differing rates of distribution and clearance. It
was found necessary to work out a new procedure for the determin-
ation of barbiturates, since none of the methods given in the
literature were found suitable for the estimation of the unstable
members of the series, such as evipan and pentothal. The method
arrived at had many advantages over previously described
techniques in the rapid, routine determination of any type of
barbiturate.

Owing to the outbreak of war, the method was never used here
for the purpose for which it was designed, but it is now in use for routine testing in a large mental hospital.

Reprint of paper enclosed.

(3c) The pharmacology of arsenic poisoning.

As a member of a Chemical Defence Extra-Mural Research Team, headed by Professors A.J. Clark and G.F. Harries, the candidate has worked on this subject since December, 1940.

To meet the requirements of this investigation, a new procedure was worked out for the micro-titrmetric estimation of arsenic in digests of biological material. Thousands of estimations involving the use of this technique have now been carried out by members of the team, with consistently satisfactory results.

The first step in studying poisoning by a gas is to determine the durations of exposure necessary to cause death at various concentrations. Results obtained by workers at Porton and by the candidate suggested that, over the time range 5 minutes to 24 hours, the concentrations of arsenic (C) and the times of exposure (T) required to produce death in 50% of a group of animals (LD₅₀) were related according to the equation:

\[ \text{LD}_{50} = k \cdot \text{T} \]

(1)

Experiments carried out with mice by the candidate indicated that below 1 minute the relationship was:

\[ T < 1 \text{ minute, } \quad C \cdot T = \text{constant.} \]

(2)
Section of application for Gunning (Victoria Jubilee) Prize in Materia Medica, submitted by G.A. Levvy.

(3c) The pharmacology of arsine poisoning.

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\[ T > 5 \text{ minutes,} \quad C^2T = \text{constant.} \quad (1) \]

Experiments carried out with mice by the candidate indicated that below 1 minute the relationship was:

\[ T < 1 \text{ minute,} \quad CT = \text{constant.} \quad (2) \]
From determinations of the arsenic contents of mice after LD$_{50}$ exposures to arsine, it seemed that the amount of gas absorbed ($U$) was proportional to both $C$ and $T$ over the whole time range investigated (15 seconds to 24 hours), i.e.:

$$U = kCT.$$  \hspace{1cm} (3)

The amount of absorbed arsine necessary to produce death in 50% of a group of mice for an exposure of 24 hours was about 25 times that required for an LD$_{50}$ exposure lasting 1 minute. This wide variation in the toxicity of a given amount of arsine according to the concentration at which it was absorbed explained the peculiar relationship between $C$ and $T$ shown in equation 1, and suggested the existence in the body of a powerful detoxicating mechanism for the gas. As expected, below 1 minute the uptake of arsine was found to be the same for different LD$_{50}$ exposures, representing the minimum quantity necessary for death, and probably the most effectively toxic fraction of arsine absorbed during longer exposures. The LD$_{50}$ uptakes of arsine ($U_{50}$) can be expressed as follows:

- $T > 5$ minutes, $U_{50} = \text{constant} \div \text{concentration}$  \hspace{1cm} (4)
- $T < 1$ minute, $U_{50} = \text{constant}$.  \hspace{1cm} (5)

The fact that, when arsine is administered in moderate concentrations, large quantities must be absorbed to produce death is of great importance in considering the possibility of its use as a war gas. Investigation of the detoxicating
mechanism is proceeding along two lines. The first of these has as its object the elucidation of the rôle played in the lethal effects of arsine by its remarkable haemolytic action. Preliminary experiments on the differential distribution of arsenic between erythrocytes and other tissues after various LD$_{50}$ exposures to arsine showed that organs contain quite large amounts of the poison, compared with red blood corpuscles, before haemolysis begins.

The second obvious line of investigation of arsine detoxication is to study the excretion of arsenic, both qualitatively and quantitatively, after administration of the gas. Analyses of mice killed at intervals after administration of arsine, urine analyses and cumulation experiments to find what fraction of a lethal dose can be given daily for a prolonged time without causing death all agree in suggesting that about half the arsenic absorbed as arsine is excreted in the first 24 hours. This rate is not even of the same order as that of the detoxication mechanism. In most of these experiments, however, the gas was administered by intraperitoneal injection, and, whilst arsine can certainly be given quantitatively in this way, there are difficulties in applying results so obtained to inhaled gas.
EMERGENCY PREPARATION OF PYROGEN-FREE WATER

BY

J. C. LEES, M.B., Ch.B.

AND

G. A. LEVY, Ph.D.

(From the Department of Pharmacology, University of Edinburgh)

The production of distilled water free from pyrogens requires the special precautions regarding distillation and autoclaving laid down in the monograph in the British Pharmacopoeia Addendum, 1936. A civil or military emergency may give rise to a sudden demand for very large quantities of intravenous saline. Here is described a method whereby tap-water or unsatisfactory distilled water may be readily freed from pyrogens, even though only the most primitive laboratory facilities are available.

Pyrogens were first described by Hort and Penfold (1911). Their exact nature was thoroughly established by the work of Seibert et al. (1923, 1925; Bourn and Seibert, 1925). Banks (1934) and Co Tui et al. (1936, 1937, 1939) have also carried out research on this subject, and many other American authors have studied pyrogens from a clinical point of view (Walter, 1935, 1936; Rademaker, 1930, 1933; Thompson, 1933). Although it is not yet widely enough realized, the cause of pyrexial reactions following intravenous injection has been definitely established, and many of the traditionally accepted factors have been proved to be of relatively little importance. Pyrogenic agents are filterable thermostable products of the growth of certain strains of bacteria that are ubiquitous enough to infect any water that is not kept absolutely sterile. These bacteria grow very rapidly in distilled water, and large quantities of pyrogen can be formed in a day in water kept at room temperature. Ordinary autoclaving does not destroy pyrogens, but water can be freed from them by distillation.

To obtain pyrogen-free water the still used must be fitted with vapour baffles to prevent passage of droplets,
and the condensing system should be of such a design that it can readily be kept scrupulously clean. All glassware must be carefully cleaned immediately before use, and for storage the water (or solutions prepared from it) should be autoclaved and sealed very shortly after distillation.

Importance of Pyrogens

A typical pyrogenic reaction consists of a rigor and feeling of chill any time from fifteen minutes to eight hours after the injection. There is a sharp rise in temperature and pulse rate. The reaction is followed by profuse sweating and a fall in temperature. There may also be nausea, vomiting, headache, and albuminuria.

It is difficult to estimate the amount of damage done by these reactions. They are often stated to be relatively harmless, but surgeons and physicians agree that they are a highly undesirable toxic side-action of saline therapy. Owing to the fact that a considerable proportion of patients who receive saline infusions are in a dangerous condition, it is not possible to estimate how far pyrogenic reactions may be an actual cause of death.

Testing for Pyrogens

Seibert and Mendel (1923) and Banks (1934) have described a satisfactory technique for the detection of pyrogens by measuring the increase in rectal temperature produced by intravenous injection of the solution into rabbits. Stringent precautions are necessary to ensure that the temperatures are unaffected by any other factors throughout the experiments. Our method of testing differed from that given by these authors only in that we used larger numbers of animals for each test and took 20 c.c.m. as a standard volume of normal saline for injection—corresponding roughly to 500 c.c.m. in an adult human.

A positive reaction is shown by a sudden sharp rise in temperature of 1° to 4° F. within two hours of the injection, the temperature returning to normal within four hours. The response may be estimated in two ways: (a) Average (for all animals injected) of the difference between the highest temperature recorded after injection and the highest temperature recorded before injection for each rabbit. (Seibert measured the differences between the highest and lowest temperatures recorded throughout the entire day, but we found that the measurement stated gave more reliable results.) (b) Determination of
the number of positive reactions by inspection of the temperature charts of the animals used.

It should be noted that the amount of organic impurity in water as found by the permanganate test is not necessarily an index of the pyrogen content, although it has been used for this purpose by some authors.

Reliability of Test

Considerable care and experience are required in carrying out the test, as the rabbit’s temperature is readily affected by excitement. We found, however, that the method gave very consistent and reliable results in distinguishing pyrogenic from non-pyrogenic waters. For purposes of comparison we took as standards solutions from two distinct sources, samples of which were tested at frequent intervals over a wide period. The results are summarized in Table I.

<table>
<thead>
<tr>
<th>TABLE I.—Action of 20 c.cm. Normal Saline (Sources A and B) Injected Intravenously into Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Number of animals used</td>
</tr>
<tr>
<td>Average rise in temperature produced (°F.)</td>
</tr>
<tr>
<td>Number of positive reactions by inspection of temperature charts</td>
</tr>
</tbody>
</table>

\* For this figure the difference between the maximum temperature in the afternoon and the maximum temperature in the morning was recorded, injections being always carried out about midday.

Since the response of individual rabbits to injection of pyrogen was found to be in extent subject to the same variation as any other biological phenomenon, we considered it advisable to establish statistically the limits of accuracy of the rabbit test. We accordingly submitted the results obtained with solutions A and B to analysis. Dr. W. O. Kermack kindly advised us as to how to proceed. Analysis showed that if five rabbits were used and an average rise of 0.8° F. was taken as a positive reaction, then the chance of a rise at least as great as this occurring with a non-pyrogenic water was 1 in 20, and the chance that a pyrogenic water should give a rise equal to or less than this was also 1 in 20.

Clinical Significance of Animal Test

Various authors (Co Tui et al., 1937, 1939; Banks, 1934; Rademaker, 1930, 1933) have shown that solutions
found to be pyrogenic by testing in animals produce undesirable reactions on injection into patients, whereas no unpleasant symptoms are associated with injection of solutions found non-pyrogenic by this method. The rabbit test has been used as a control in the preparation of commercial solutions for intravenous injection that have given general satisfaction.

The pyrogenic water taken as a standard (B, Table I) was one employed for preparation of intravenous saline. Clinical experience had indicated that, while not unusable, it was unsatisfactory. The rabbit test thus provided clear information regarding pyrogens present in quantities likely to be encountered in actual clinical practice.

Removal of Pyrogens by Charcoal

That water could be freed from fever-producing substances by an adsorption process was suggested to us by the work of Co Tui et al. (1936, 1937, 1939) on the removal of pyrogens from solutions by passage through a Seitz asbestos filter. We studied the actions of the following common adsorption agents on pyrogen; aluminium oxide, kaolin, novasorb (magnesium trisilicate), kieselguhr, fullers' earth, powdered charcoal, and granular charcoal.

A constant supply of pyrogenic water was ensured by inoculating 20 litres of distilled water contained in a large glass jar with 100 c.cm. of a non-autoclaved sample of water B (Table I). The contents of the jar became strongly pyrogenic in a day or two. As water was drawn from this store distilled water was added to keep the volume at 20 litres. From time to time the water was tested for pyrogen content, which showed no signs of diminishing. Dr. J. C. J. Ives of the bacteriology department very kindly examined a sample of water from the reservoir, and from it isolated strains of bacteria which were capable of growth in distilled water and formed pyrogen.

In testing the adsorption agents it was found convenient simply to shake a weighed amount with a measured volume of the pyrogenic water. After shaking for fifteen minutes the solid was removed by means of a Jena glass filter or a fluted filter paper. Control experiments showed that filtering the pyrogenic water in no way affected its fever-producing properties.
We found that powdered charcoal was a powerful adsorbent of pyrogens, being much superior to any of the other agents examined. The following powdered charcoals were examined: ultracarbon (Merck), activated (B.D.H.), animal, wood, Norit A, blood (B.D.H.). There was little to choose between any of these in pyrogen-adsorbing power. In Table II are summarized the results obtained for the pyrogen content of the standard water after treatment with powdered charcoals. These results are of interest in relation to the preparation of non-toxic inulin by the use of charcoal (cf. Co Tui et al., 1937). The following varieties of granular charcoal were examined: ultracarbon (Merck), activated (B.D.H.), animal (B.D.H.), wood (B.D.H.), sugar (B.D.H.), coconut shell (B.D.H.). None of these removed pyrogens from water, except when part was broken down to powder. The results are shown in Table II.

As the method of testing adsorptive powers described above might not be considered a fair one for granular charcoals owing to the difficulty in bringing them into intimate contact with the liquid, and since the adsorption process is possibly an equilibrium one, these were also examined by means of adsorption towers 20 cm. long and 2 cm. in diameter. In spite of the fact that great care was taken to ensure that the liquid came into intimate contact with the charcoal, the granular charcoal failed to pick up pyrogens. The average rise in temperature produced on injection of the water after passing through the tower was 1.5° F. and the number of positive reactions noted was 12 in 13. The results thus

<table>
<thead>
<tr>
<th>Grammes of charcoal per 100 c.m. of water</th>
<th>Controls</th>
<th>Powdered Charcoal</th>
<th>Granular Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Average rise in temperature (°F.)</td>
<td>2.14</td>
<td>0.20</td>
<td>0.27</td>
</tr>
<tr>
<td>No. of positive reactions by inspection of temperature charts</td>
<td>9/9</td>
<td>1/31</td>
<td>1/16</td>
</tr>
</tbody>
</table>

parallel those obtained by the shaking technique. We were reluctant to come to the conclusion that granular charcoal was completely useless for this purpose, as the possibility had been entertained of making use of it in
an automatic pyrogen-removing filter. It was found possible to remove pyrogens from large quantities of water by forcing under pressure through a bed of powdered charcoal. This method, however, seems to have no advantage over the use of large-scale Seitz asbestos filters.

The results show that a strongly pyrogenic water or solution may be freed from pyrogens by the simple procedure of shaking for a few minutes with a very small quantity (0.1 per cent.) of powdered charcoal. This quantity of charcoal can be readily removed by decanting through a fluted filter paper. The receiving vessel, which must be spotlessly clean, should be washed out with a little of the filtrate before collecting the remainder. Although there seems to be little to choose between different charcoals as regards efficiency in picking up pyrogens, from the point of view of cheapness, availability, and chemical cleanliness B.D.H. activated powder seems the most suitable for this purpose.

**Summary**

Shaking for fifteen minutes with powdered charcoal (1 part per 1,000) removes pyrogens from heavily contaminated water. The charcoal can be readily separated by decanting through a filter paper. This provides a simple emergency method for obtaining water suitable for preparation of intravenous infusions.

We desire to express our thanks to Professor A. J. Clark for his continued interest and advice in carrying out this research. The expenses of the work were defrayed from the Moray and Crichton (J.C.L.) Funds of Edinburgh University.

**References**

--- (1925). Ibid., 71, 621.
13. THE ESTIMATION OF BARBITURATES IN BLOOD

By G. A. LEVY

From the Department of Pharmacology, University of Edinburgh

(Received 25 November 1939)

In connexion with a study of the distribution and fate in the body of the shorter-acting barbiturates, evipan and pentothal, it was necessary to carry out estimations of these drugs in blood and other biological media. The quantitative extraction of these unstable compounds presents some difficulty, and none of the methods given in the literature for dealing with more stable barbituric acids [Brundage & Gruber, 1937; Fischer, 1939; Herwick, 1933; Linegar et al. 1935; Koppanyi et al. 1934] was found suitable. It was thus necessary to work out a satisfactory procedure.

In clinical work the need has long been felt for a simple method of determining barbiturate concentrations in blood. The method described here, in addition to giving satisfactory extraction of evipan or pentothal, has many advantages over previously described methods in the rapid, routine determination of any type of barbituric acid in blood. It was found that 20 ml. blood from a patient on only a moderate dosage of barbitone were quite sufficient for examination. A study has been planned of the use of the procedure as a routine clinical test. The method should also be of value in toxicology.

For quantities of 1 mg. or more of barbiturate, the observed maximum error for the complete procedure is 20%, and for smaller quantities the error is correspondingly greater. This large experimental error is to be expected, since the colorimetric estimation after extraction is itself only accurate to within 10%, and since the drugs in question are unstable and difficult to obtain absolutely pure. The method is considered sufficiently accurate to meet the requirements of the pharmacological research for which it was designed, and it is felt that efforts to determine evipan or pentothal more accurately would defeat their own end, owing to the instability of these compounds. Within the limits given, the method is very reliable; three determinations may be carried out in 5 hr., and require no attention for much of that time.

Some experiments have been carried out on the extraction of barbiturates from tissues, other than blood, with promising results. The technique may be applicable to the extraction of drugs other than barbiturates.

Method

Evipan and pentothal are completely insoluble in acid and neutral solutions, and in alkaline solution they are extremely unstable; hence no method of extraction involving a protein precipitation can be used. Methods of direct liquid extraction from acid media with large volumes of a fat solvent can hardly be considered suitable for a routine quantitative estimation. The technique finally adopted was to dry the blood with anhydrous sodium sulphate so as to give a fine powder which could be extracted in a Soxhlet apparatus with a fat solvent. After purification, the barbiturate was then estimated by Koppanyi's colour reaction with cobalt acetate and isopropylamine in chloroform.
While this work was in progress, Griffon & Breton [1939] published a method for the detection of barbiturates, involving the use of sodium sulphate as a drying agent. These authors recommend as a qualitative test for barbituric acids a modification of Koppanyi's reaction, using diethylamine in alcohol in place of isopropylamine in chloroform; this has been examined and found to be inferior to the original for quantitative work.

To acidify the blood before drying, sodium dihydrogen phosphate was added. The use of this has the advantage that no neutralization of the extract is necessary, nor is there any release of pigments from the blood as with strong acids. It was considered desirable to carry out the extraction at as low a temperature as possible, and ether was therefore at first used as solvent, but the fats extracted from the dried blood were found to interfere in the colour reaction. This difficulty was overcome by using the mixture of equal parts of ether and light petroleum (b.p. 30–40°), which Brundage & Gruber [1937] recommend for the elution of barbiturates from charcoal. With this solvent a crystalline residue practically free from fat was obtained.

The barbiturate thus extracted from blood could, in most cases, be taken up straight away in chloroform and estimated, but occasionally it was contaminated by a small quantity of phospholipins; this contamination always occurred with extracts of tissues other than blood. The contaminants must be removed to obtain a true colour match with the standard, and, as a routine precaution, all ethereal extracts were shaken with a mixture of charcoal and magnesium oxide [Fischer, 1939] for a few minutes before evaporation. Great care must be exercised in the use of an adsorbing agent for the purification of a barbiturate solution. Even under the conditions specified below for purifying extracts there was found to be a very slight, but quite definite, adsorption of the drug from a barbiturate solution in ether and light petroleum. Unfortunately, no alternative scheme of purification was found practicable.

![Fig. 1. Recoveries of barbitone from blood for different times of extraction.](image-url)
ESTIMATION OF BARBITURATES

All ether used in the estimation must be freed from oxides, as the smallest traces of these prevent the reaction with cobalt acetate, and they are not removed in the treatment with charcoal and magnesium oxide. Estimations can best be carried out serially.

Fig. 1 shows the relation found between time of extraction and percentage recovery of 10 mg. sodium barbitone added in 1–2 ml. water to 20 ml. citrated blood. From this it is seen that extraction is complete, within the experimental error of the colorimetric estimation, in 2.5–3 hr. Barbitone being one of the least soluble of the barbiturates in the solvent used, all extractions were continued for this time.

EXPERIMENTAL

Reagents

Extraction mixture. Oxide-free ether is prepared weekly by washing with ferrous sulphate solution and water, and distilling into a bottle containing clean copper wire. It is mixed before use with an equal volume of light petroleum (b.p. 30–40°) in a measuring cylinder, the walls of which retain any water that may separate.

Charcoal and magnesium oxide mixture. The charcoal (Merck's "Ultra-carbon") is washed with oxide-free ether containing a few drops of glacial acetic acid, and then with ether till free from acid. Three parts prepared charcoal are mixed with one part magnesium oxide.

Cobalt acetate solution. 1% in absolute methyl alcohol.

isoPropylamine solution. 5% by volume in absolute methyl alcohol.

Standard solutions for colorimeter. Solutions of the barbituric acid (not the sodium salt) under examination are prepared in chloroform in the following concentrations: 0.02, 0.04, 0.06, 0.08, 0.10, 0.12%. These are stored in dark, glass-stoppered bottles.

Procedure

In a mortar of 250 ml. capacity, 20 ml. blood (coagulated or citrated) is mixed with 2 g. NaH₂PO₄. There are then added, in small portions with constant grinding, 40 g. anhydrous Na₂SO₄. After thorough mixing of blood and sulphate, the mortar is placed in a desiccator for 10–15 min. to allow complete drying to occur. The dry cake obtained is broken up and packed into a 100 ml. Soxhlet thimble. It is advisable to place a filter paper in the top of the filled thimble to prevent displacement of the powder. Extraction is carried out for 2.5–3 hr. at 50° with 80 ml. of ether-petroleum mixture. The addition of a few glass beads to the ether is necessary to facilitate even boiling.

At the end of the extraction the contents of the flask are shaken for a few minutes with about 0.25 g. charcoal and magnesium oxide. This is then removed by filtration directly into a distilling flask through a Jena sintered glass funnel, washing with small quantities of ether-petroleum, and the filtrate is distilled at 50°. The crystals of barbiturate which remain in the flask are taken up in a small quantity of chloroform and transferred to a 10 ml. graduated centrifuge tube, the solution being made up to a convenient volume. Owing to the difficulty of measuring chloroform, the loss of accuracy involved in the use of graduated tubes for this purpose was considered to be justified by the gain in rapidity of working.

To 2 ml. of the chloroform solution are added 0.1 ml. cobalt acetate solution and 0.6 ml. isoPropylamine solution, and the colour is compared in a micro-
colorimeter with that given by 2 ml. of one of the standard solutions similarly treated. It may be necessary to dilute or concentrate the unknown solution to bring it within the range of concentrations covered by the standards.

Results

Figures are shown in Table 1 for the recoveries of four barbituric acids added, as their sodium salts in 1 ml. water, to 20 ml. citrated blood. Blank determinations were carried out, using double quantities of citrated or fresh coagulated blood to which no barbiturate had been added. After distilling off the ether-petroleum mixture, the flask was washed out either with 2 ml. chloroform or with one of the standard barbiturate solutions. No colour was produced with cobalt acetate and isopropylamine in the former case, and in the latter no change was observed in the intensity or shade of the colour given by the standard solution. From the latter series of observations it appears that, under the stated conditions, there is extracted from blood no substance which could cause a false result through an additive or potentiating effect on the reaction between barbiturates and cobalt acetate.

<table>
<thead>
<tr>
<th>Barbiturate</th>
<th>Added mg.</th>
<th>Found mg.</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbitone</td>
<td>10.4</td>
<td>9.5</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>8.9</td>
<td>8.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>4.6</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>1.05</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>0.46</td>
<td>124</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>10.3</td>
<td>8.5</td>
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</tr>
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<td>5.1</td>
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</tr>
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<td></td>
<td>0.51</td>
<td>0.41</td>
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</tr>
<tr>
<td>Pentothal</td>
<td>9.3</td>
<td>7.8</td>
<td>84</td>
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<td></td>
<td>4.6</td>
<td>4.3</td>
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<td>0.46</td>
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<td>9.3</td>
<td>94</td>
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<td>8.7</td>
<td>7.2</td>
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</tbody>
</table>

Samples of blood (supplied by Dr H. Tod) from three patients, to whom barbitone had been regularly administered for years, were examined by this method (Table 2). The dosage was such as had been found to give the same degree of depression in each case.

<table>
<thead>
<tr>
<th>Case</th>
<th>Daily dose mg./kg.</th>
<th>Barbitone found mg./20 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mrs G.</td>
<td>20.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Miss D.</td>
<td>16.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Miss S.</td>
<td>16.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Summary

A simple procedure has been developed for the extraction of unstable barbiturates from blood. It has been found satisfactory for use in the routine determination of any type of barbiturate.
The author desires to express his gratitude to Prof. A. J. Clark for his continued interest and advice, to Dr P. Eggleton for many valuable suggestions, and to Imperial Chemical Industries Ltd. for defraying the expenses of this work.

REFERENCES

INTRODUCTION

A large number of $\beta$-amino-ketones, of the general formula

$$R' \cdot CO \cdot CH_2 \cdot CH_2 \cdot NR_2, HCl$$

where $R' = \text{phenyl}, \text{alkoxyphenyl}, \text{pyrryl-2}, \text{thienyl-2},$ and $NR_2 = \text{piperidino}, \text{dimethylamino}, \text{diethylamino},$ have been found to possess local anaesthetic properties (1, 2, 3). Unfortunately, the degree of irritation is usually high with such compounds.

To study the effect of introducing thiazole and furane nuclei into the molecule at $R'$, heterocyclic ketones of types I and II, respectively, were synthesised (3) by a general reaction for the preparation of $\beta$-amino-ketones (4). As many compounds of each type as possible were prepared by variation of the dialkylamino residue, $NR_2$.

$$NR_2 = NC_6H_{10}, N(CH_3)_2, N(C_2H_5)_2, N(n-C_3H_7)_2.$$

$$NR_2 = NC_6H_{10}, N(CH_3)_2, N(n-C_3H_7)_2, N(n-C_4H_9)_2, N(CH_2\cdot CH_2OH)_2.$$
A group of pyrazolines, of the general formula

\[
\begin{align*}
R' & = \text{phenyl, substituted phenyl, furyl-2, and } NR_2 = \text{piperidino, dialkylamino, have been studied by H. K. Sinha}\ (5, 6). \\
\text{The majority possess strong local anaesthetic activities, while their toxicities compare favourably with that of cocaine, and the irritation caused is slight.}
\end{align*}
\]

With a view to studying the effect of introducing various \( o-n \)-alkoxyl groups into a substituent phenyl radical at position 5, four pyrazolines of type IV were prepared by isomerisation of the phenylhydrazones of unsaturated \( \beta \)-piperidino-ketones (III). Preliminary tests on one of these compounds (IV, Alk = \( n - C_4H_9 \)) were carried out by Sinha (6).

\[
\begin{align*}
\text{III} & \\
\text{IV} & \\
\text{Alk} & = \text{CH}_3, \text{C}_2\text{H}_5, n-C_3\text{H}_7, n-C_4\text{H}_9.
\end{align*}
\]

An examination of the local anaesthetic properties of the compounds, types I, II, III and IV, was carried out as follows. Details of the methods are given by Sinha (5). The local anaesthetic efficiency of a drug was taken to be the reciprocal of the minimum
concentration producing an action of at least 10 minutes duration, the result being expressed as the relative potency compared with cocaine.

**LOCAL ANAESTHESIA OF RABBIT’S CORNEA**

All the compounds were first tested by this method, which measures the power of a drug to penetrate mucous membranes and to paralyse nerve-endings. Any irritation caused by the concentrations used was carefully noted.

In groups I and II, only one compound (I, NR₂ = N(CH₃)₂) was found to be appreciably stronger than cocaine, and all proved to be highly irritant, increase in the size of the dialkylamino group (NR₂) being associated with increased irritation. Compounds of type III also caused irritation. In this group, increase in activity, up to three times that of cocaine, was observed as the size of the alkoxy group was increased.

The results obtained for the pyrazolines (IV) are given in table 1. In three cases (Alk = CH₃, C₃H₇, C₄H₉), the anaesthesia produced by the concentrations shown lasted for about 30 minutes. Lower concentrations, however, had no effect at all, and increasing the concentrations did not appreciably increase the durations of action. No irritation was observed with any member of this group.

Only the compounds of type IV were considered worthy of further examination, the others being too irritant to be of value.

**LOCAL ANAESTHESIA OF HUMAN WHEAL**

By intradermal injection, the power to paralyse nerve-endings may be measured, independently of the ability to penetrate protective tissues.
In table 2 are recorded the results obtained by this method for the pyrazolines of type IV. The greatest possible concentration of the propoxy-compound (Alk = C₆H₅) in isotonic saline was 0.1 per cent. This caused, on the average, 5 minutes anaesthesia. For this duration, its potency equals that of cocaine. No irritation was caused by any of the pyrazolines in this test.

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<thead>
<tr>
<th>ALK</th>
<th>CH₃</th>
<th>C₆H₅</th>
<th>C₃H₇</th>
<th>COCAINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration per cent causing:</td>
<td>0.01</td>
<td>0.01</td>
<td>0.0067</td>
<td>0.33</td>
</tr>
<tr>
<td>10 minutes anaesthesia.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative potency compared with cocaine</td>
<td>33</td>
<td>33</td>
<td>50</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Dose</th>
<th>Number of mice injected</th>
<th>Number of deaths</th>
<th>Mortality per cent</th>
</tr>
</thead>
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</tr>
<tr>
<td></td>
<td>2.0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>10</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>11</td>
<td>6</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>1-Phenyl-3-(α-piperidinoethyl)-5-(2'-methoxy-phenyl)-pyrazoline hydrochloride</td>
<td>1.5</td>
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<tr>
<td></td>
<td>3.0</td>
<td>5</td>
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<td>5.0</td>
<td>5</td>
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<td></td>
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<td>10</td>
<td>1</td>
<td>10</td>
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<tr>
<td></td>
<td>4.0</td>
<td>15</td>
<td>4</td>
<td>27</td>
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<td></td>
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<td>5</td>
<td>100</td>
</tr>
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<td>1-Phenyl-3-(α-piperidinoethyl)-5-(2'-n-butoxy-phenyl)-pyrazoline hydrochloride</td>
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<td>5</td>
<td>0</td>
<td>0</td>
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<td>2.5</td>
<td>5</td>
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<td>80</td>
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TOXICITY

Median lethal doses were determined by intraperitoneal injections into mice, of average weight 20 grams, 0.2 per cent solutions being used. The doses were varied by varying the volume of fluid, and were calculated in milligrams per 20 grams body-weight.

The three most potent members of group IV (Alk = CH₃, C₃H₇, C₄H₉) were examined; the results obtained for these and for cocaine are summarised in table 3. From these figures, approximate values for the median lethal doses may be found (table 4).

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were caused by cocaine, were not observed on injection of any of the pyrazolines.

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<th>CH₃</th>
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<th>C₃H₇</th>
<th>C₄H₉</th>
<th>COCAINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.L.D.</td>
<td>3.2</td>
<td>4.5</td>
<td>4.5</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Relative toxicity compared with cocaine</td>
<td>0.9</td>
<td>0.6</td>
<td>0.6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Therapeutic value—cornea</td>
<td>3.3</td>
<td>5</td>
<td>3.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Therapeutic value—wheat</td>
<td>37</td>
<td>55</td>
<td>83</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

THERAPEUTIC VALUES

Having found their relative toxicities compared with cocaine, the therapeutic values of the pyrazolines may be calculated (table 4), according to the formula

\[
\text{therapeutic value} = \frac{b}{a} \cdot \frac{x}{y}
\]

where \(a\) = effective concentration of drug, \(b\) = effective concentration of cocaine, \(x\) = median lethal dose of drug, and \(y\) = median lethal dose of cocaine.

DISCUSSION

On testing on the rabbit's cornea, \(\beta\)-amino-ketones containing thiazole and furane nuclei (I and II) have been found to be too
irritant to have any value as local anaesthetics. Variation of R' and NR₂ in ketones of the type R':CO·CH₂·CH₂·NR₂ has thus failed to eliminate this undesirable property.

The 1:3:5-trisubstituted-pyrazolines (IV) studied were, on the other hand, not at all irritant. With one exception (Alk = C₃H₇), they were more potent than cocaine in producing local anaesthesia of the rabbit's cornea, and were very powerful when injected intracutaneously. The toxicities of the three active members of the group were found to be less than that of cocaine. From a comparison of the results with the figures given by Sinha for other similar pyrazolines, it appears that the introduction of an o-alkoxyl group into the 5-phenyl nucleus enhances the local anaesthetic properties of the 1:3:5-trisubstituted-pyrazoline molecule as a whole. The therapeutic values of those compounds examined shows that the combination of desirable properties is greatest in the case of the 2'-n-butoxyphenyl-pyrazoline (Alk = C₃H₇).

For purposes of comparison, the unsaturated ketones (III) from which the pyrazolines were prepared were also tested. Like the other ketones examined, however, these compounds, although local anaesthetics, were too irritant to be of value.

SUMMARY

A study of β-amino-ketones containing thiazole and furane nuclei by the corneal method has shown them to be too irritant to have any value as local anaesthetics. Four pyrazoline derivatives, of a type already known to possess local anaesthetic properties, were examined more extensively. Three of these were more potent and less toxic than cocaine, and were not at all irritant. The introduction of an o-n-butoxy-group into one of the substituent phenyl nuclei appears to have a beneficial effect on the properties of the molecule as a whole. Local anaesthesia was also caused by the unsaturated piperidino-ketones from which the pyrazolines were prepared, but they proved to be more irritant than cocaine.
The authors desire to express their gratitude to Prof. A. J. Clark for his advice throughout the course of this work. Thanks are also due to Lord Suffolk for his assistance in carrying out the toxicity tests, and to those who volunteered for intradermal injections. A Maintenance Grant from the Department of Scientific and Industrial Research is gratefully acknowledged by one of us (G. A. L.).

REFERENCES

(1) Mannich and Lammering: Ber., 55, 3510, 1922.
(2) Bricke and Blake: J. A. C. S., 52, 235, 1930.
(3) Levy and Nisbet: J. C. S., 1053, 1938.
(4) Mannich and Heilner: Ber., 55, 356, 1922.
(6) Sinha: Ibid.
INTRODUCTION

A large number of β-amino-ketones, of the general formula

\[ R' \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{NR}_2 \cdot \text{HCl} \]

where \( R' \) = phenyl, alkoxyphenyl, pyrryl-2, thienyl-2, and \( \text{NR}_2 \) = piperidino, dimethylamino, diethylamino, have been found to possess local anaesthetic properties (1, 2, 3). Unfortunately, the degree of irritation is usually high with such compounds.

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\[ \text{NR}_2 = \text{NC}_5\text{H}_{10}, \text{N(CH}_2\text{CH}_2\text{OH})_2. \]
A group of pyrazolines, of the general formula

\[
R'\text{--CH--CH}_2\text{--CH}_2\text{--CH}_2\cdot\text{NR}_2\cdot\text{HCl}
\]

where \( R' \) = phenyl, substituted phenyl, furyl-2, and \( \text{NR}_2 \) = piperidino, dialkylamino, have been studied by H. K. Sinha (5, 6). The majority possess strong local anaesthetic activities, while their toxicities compare favourably with that of cocaine, and the irritation caused is slight.

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\[
\text{OAlk}\quad\text{CH:CH\cdotCO\cdotCH}_2\cdot\text{CH}_2\cdot\text{NC}_5\text{H}_{10}\cdot\text{HCl}
\]

III

\[
\text{OAlk}\quad\text{CH--CH}_2\text{--CH}_2\cdot\text{NC}_5\text{H}_{10}\cdot\text{HCl}
\]

IV

Alk = \( \text{CH}_3, \text{C}_2\text{H}_5, n\text{-C}_7\text{H}_7, n\text{-C}_4\text{H}_9 \).

An examination of the local anaesthetic properties of the compounds, types I, II, III and IV, was carried out as follows. Details of the methods are given by Sinha (5). The local anaesthetic efficiency of a drug was taken to be the reciprocal of the minimum
HETEROCYCLIC COMPOUNDS AS ANAESTHETICS

concentration producing an action of at least 10 minutes duration, the result being expressed as the relative potency compared with cocaine.

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<th>C₃H₇</th>
<th>C₄H₉</th>
<th>COCAINE</th>
</tr>
</thead>
</table>
| Concentration per cent causing:  
10 minutes anaesthesia.................. | 0.1 | 0.1  | 0.33 | 0.16 | 0.33    |
| Relative potency compared with cocaine... | 3   | 3    | 1    | 2    | 1       |

in activity, up to three times that of cocaine, was observed as the size of the alkoxy group was increased.

The results obtained for the pyrazolines (IV) are given in table 1. In three cases (Alk = CH₃, C₃H₇, C₄H₉), the anaesthesia produced by the concentrations shown lasted for about 30 minutes. Lower concentrations, however, had no effect at all, and increasing the concentrations did not appreciably increase the durations of action. No irritation was observed with any member of this group.

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<tbody>
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<td>Concentration per cent causing:</td>
<td></td>
<td></td>
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<tr>
<td>10 minutes anaesthesia</td>
<td>0.01</td>
<td>0.01</td>
<td>0.0067</td>
<td>0.33</td>
</tr>
<tr>
<td>Relative potency compared with cocaine</td>
<td>33</td>
<td>33</td>
<td>50</td>
<td>1</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>DRUG</th>
<th>DRUG Dose</th>
<th>NUMBER OF MICE INJECTED</th>
<th>NUMBER OF DEATHS</th>
<th>MORTALITY</th>
</tr>
</thead>
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<tr>
<td></td>
<td>mgm./</td>
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</tr>
<tr>
<td></td>
<td>20 grams</td>
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<td>Cocaine</td>
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<td></td>
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<td>11</td>
<td>6</td>
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Several post-mortems were performed in the course of these experiments to ensure that death had not resulted from accidental damage of abdominal organs. Convulsions, such as

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</tr>
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<tbody>
<tr>
<td>M.L.D.</td>
<td>3.2</td>
<td>4.5</td>
<td>4.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Relative toxicity compared with cocaine</td>
<td>0.9</td>
<td>0.6</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>Therapeutic value — cornea</td>
<td>3.3</td>
<td>5</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>Therapeutic value — wheal</td>
<td>37</td>
<td>55</td>
<td>83</td>
<td>1</td>
</tr>
</tbody>
</table>

were caused by cocaine, were not observed on injection of any of the pyrazolines.

THERAPEUTIC VALUES

Having found their relative toxicities compared with cocaine, the therapeutic values of the pyrazolines may be calculated (table 4), according to the formula

\[ \text{therapeutic value} = \frac{b}{a} \cdot \frac{x}{y} \]

where \( a \) = effective concentration of drug, \( b \) = effective concentration of cocaine, \( x \) = median lethal dose of drug, and \( y \) = median lethal dose of cocaine.

DISCUSSION

On testing on the rabbit's cornea, \( \beta \)-amino-ketones containing thiazole and furane nuclei (I and II) have been found to be too
irritant to have any value as local anaesthetics. Variation of R' and NR₂ in ketones of the type R'·CO·CH₂·CH₂·NR₂ has thus failed to eliminate this undesirable property.

The 1:3:5-trisubstituted-pyrazolines (IV) studied were, on the other hand, not at all irritant. With one exception (Alk = C₃H₇), they were more potent than cocaine in producing local anaesthesia of the rabbit's cornea, and were very powerful when injected intracutaneously. The toxicities of the three active members of the group were found to be less than that of cocaine. From a comparison of the results with the figures given by Sinha for other similar pyrazolines, it appears that the introduction of an o-alkoxy group into the 5-phenyl nucleus enhances the local anaesthetic properties of the 1:3:5-trisubstituted-pyrazoline molecule as a whole. The therapeutic values of those compounds examined shows that the combination of desirable properties is greatest in the case of the 2'-n-butoxyphenyl-pyrazoline (Alk = C₄H₉).

For purposes of comparison, the unsaturated ketones (III) from which the pyrazolines were prepared were also tested. Like the other ketones examined, however, these compounds, although local anaesthetics, were too irritant to be of value.

**SUMMARY**

A study of β-amino-ketones containing thiazole and furane nuclei by the corneal method has shown them to be too irritant to have any value as local anaesthetics. Four pyrazoline derivatives, of a type already known to possess local anaesthetic properties, were examined more extensively. Three of these were more potent and less toxic than cocaine, and were not at all irritant. The introduction of an o-n-butoxy-group into one of the substituent phenyl nuclei appears to have a beneficial effect on the properties of the molecule as a whole. Local anaesthesia was also caused by the unsaturated piperidino-ketones from which the pyrazolines were prepared, but they proved to be more irritant than cocaine.
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