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May 20, 19 31

I hereby recommend that the thesis prepared under my supervision by Elizabeth Shelow

entitled The Near Infra-Red Absorption Spectra of Ergosterol and Irradiated Ergosterol

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

Albert P. Mathews

George Sperti

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THE NEAR INFRA-RED ABSORPTION SPECTRA

of

ERGOSTEROL AND IRRADIATED ERGOSTEROL

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1931

by

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INFRA-RED ABSORPTION CURVES

THE NEAR INFRA-RED ABSORPTION SPECTRA OF ERGOSTEROL AND IRRADIATED ERGOSTEROL

INTRODUCTION

The physiological importance of the anti-ricketic vitamin has led to a tremendous amount of work on the structure of the parent substance, ergosterol, and upon the nature of the change of ergosterol to vitamin D. Because the infra-red spectrum of a compound depends upon the chemical linkages of the molecule, a study of the infra-red spectra of ergosterol and of irradiated ergosterol offers a promising method of attack on the problem of the nature of vitamin D.

REVIEW OF THE LITERATURE

The parent substance of vitamin D.

There is no more fascinating chapter in the history of biochemistry than that which establishes the relationship of vitamin D to rickets and the relationship of ergosterol to vitamin D.

Huldschinsky[1] in 1919 clearly demonstrated that sunlight and the radiations of the mercury vapor arc, "an artificial sun," were effective in the treatment of rickets in infants. The presence in cod liver oil of a definite antiricketic vitamin was first demonstrated by McCollum and his coworkers[2] in 1922. Goldblatt and Soames,[3] without recognizing vitamin D as distinct from "fat soluble A", found that the livers of irradiated ricketic rats protected rats on a rickets-producing diet. In 1924 Hess[4] and Steenbock[5] almost simultaneously announced that the feeding of irradiated food was as effective as irradiating the ricketic animal itself.

The antiricketic activity of cod liver oil was found to be a property of the non-saponifiable fraction, of which cholesterol is the chief constituent.[6] Cholesterol was present in all of the activatable

substances examined, and apparently pure cholesterol, many times recrystallized and of constant melting point, acquired marked antiricketic potency when irradiated. It was natural to conclude that this sterol was the precursor of vitamin D.[7, 8, 9]

After much work had been done on cholesterol and its derivatives this conclusion was questioned. Three independent communications which appeared late in 1926 stated that the activity of irradiated cholesterol is due to an impurity which persists after the usual methods of purification.

Rosenheim and Webster[10] noted that cholesterol which had been treated with charcoal or purified by conversion to the dibromide could not be activated, and also that it no longer possessed a characteristic absorption spectrum in the ultra-violet. They suggested that an impurity, "vitasterol", was responsible both for the property of developing antiricketic potency and for the characteristic absorption.

Cholesterol shows less marked ultra-violet absorption after purification. Heilbron, Morton and Kamm,[11] however, detected the characteristic absorption bands in cholesterol which had been purified by recrystallizing twenty or more times. By means of fractional crystallization they succeeded in separating the impurity and in

demonstrating that this substance shows only general absorption in the ultra-violet region. Changes in the bands during irradiation led these investigators to suggest that the impurity responsible for the characteristic absorption spectrum was closely related to vitamin D.

Pohl[12] reported that cholesterol purified by Windaus by the dibromide method showed only general absorption and could not be rendered antiricketic. Hess and his coworkers[13] also pointed out that only a very small part of the cholesterol could possibly be affected; the development of antiricketic potency and the decrease in ultra-violet absorption between 280 mu and 300 mu were apparently the only changes brought about by irradiation.

Windaus and Hess[14] and Rosenheim and Webster[15] almost simultaneously suggested that the properties of the active contaminant correspond with those of ergosterol, which Tanret[16] had isolated from ergot and described in 1889. Rosenheim[17] had previously reported that irradiated ergosterol was very potent. That ergosterol is the parent substance of vitamin D has since been substantiated by many workers.

There is some evidence that substances other than ergosterol may be rendered antiricketically act-

ive. Bills and Honeywell[18] found that cholesterol which had been treated with potassium permanganate, with charcoal, or by bromination and subsequent reduction, could still be activated. The potency developed is difficult to account for on the basis of traces of ergosterol surviving the purification process.

Jendrassik and Kemenyffi[19] reported that cholesterol can be activated after purification by the dibromide method.

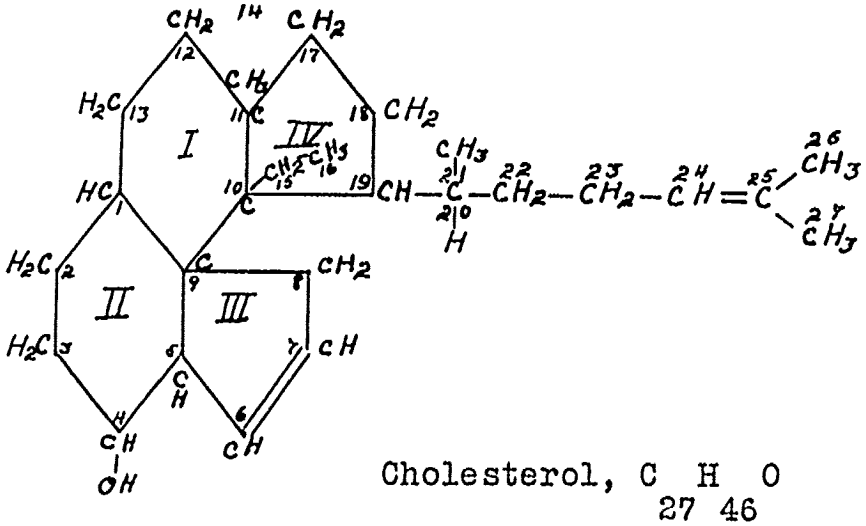
Koch, Koch and Lemon[20] found that cholesterol purified either by way of the dibromide or by treating with potassium permanganate showed only general absorption in the ultra-violet region, although it retained 1/30 to 1/70 of its antiricketic value. Purified products heated slightly above the melting point under conditions which avoid oxidation show an increase in activatability. The heated cholesterol shows strong general absorption in the ultra-violet, but no bands. These investigators believe that this activatability is not due to traces of ergosterol but that it may be a property of various sterols or of certain forms of these sterols.

If activatability is a property of compounds of certain configuration rather than of one particular sub-

stance, other substances with a molecular configuration that can be activated may possibly be found. It is certain that vitamin D has the typical sterol grouping and that it develops either from ergosterol or from some substance having the same ultra-violet absorption. There can be no doubt that ergosterol is a parent substance of vitamin D; the bulk of the evidence favors the present view that it is the sole provitamin D.

The Chemistry of Ergosterol

Ergosterol was identified as a member of the sterol group by Tanret. Important contributions to our knowledge of the sterols have been made by Windaus[21] and Wieland[23]. Ergosterol, $C_{27}H_{42}O$, with three double bonds, is closely related to cholesterol, $C_{27}H_{46}O$, with one double bond. Wieland proposes this formula for cholesterol:



Cholesterol, $C_{27}H_{46}O$

The positions of the two additional double bonds in ergosterol have never been definitely established. Heilbron, Morton and Sexton[23] undertook a study of the ultra-violet absorption spectra of ergosterol, cholesterol and derivatives with a view of correlating unsaturation and ultra-violet absorption in the sterol group. They found that selective absorption occurs only when at least two double bonds are present in the molecule. Ergosterol is characterized by well-

defined absorption bands at 293.5, 281.5, and 270 mμ; its absorption spectrum closely resembles that of cholesterilene. The maximum absorption for each of the three bands of ergosterol is, however, about 250 Angstrom units farther in the ultra-violet, and the molecular extinction coefficients are of the order: ergosterol, 10,000; cholesterilene, 2,400. This shift of the absorption bands toward the ultra-violet and the greatly increased extinction coefficient of ergosterol, due to the introduction of an additional double bond, are quite in harmony with the general views on absorption spectra. On the basis of ultra-violet absorption they infer that of the three double bonds in ergosterol, two occupy the same positions as do the two double bonds of cholesterilene.

The constitution of cholesterilene is therefore of interest. Because the literature shows many discrepancies in the physical constants and chemical behavior of compounds described as cholesterilene, the question was re-examined by Heilbron and his coworkers. 24-25

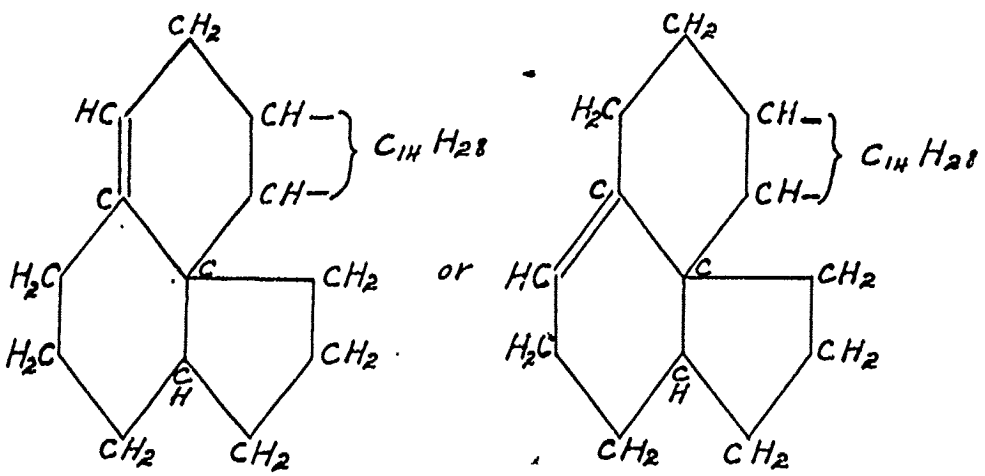
Pseudo-cholestene is formed, along with cholesterilene, by the dry distillation of cholesterol. Although there are conflicting opinions, Heilbron believes that the formation of pseudo-cholestene is due to two distinct reactions:

(a) The dehydration of part of the cholesterol to cholesterilene,

(b) The dehydrogenation of a further part to cholestenone.

Reaction (b) takes place more readily than (a), the hydrogen evolved being used to hydrogenate cholesterilene to cholestene. The dehydrogenation of the secondary alcohol group is regarded as the fundamental reaction which takes place during the decomposition of cholesterol by heat.

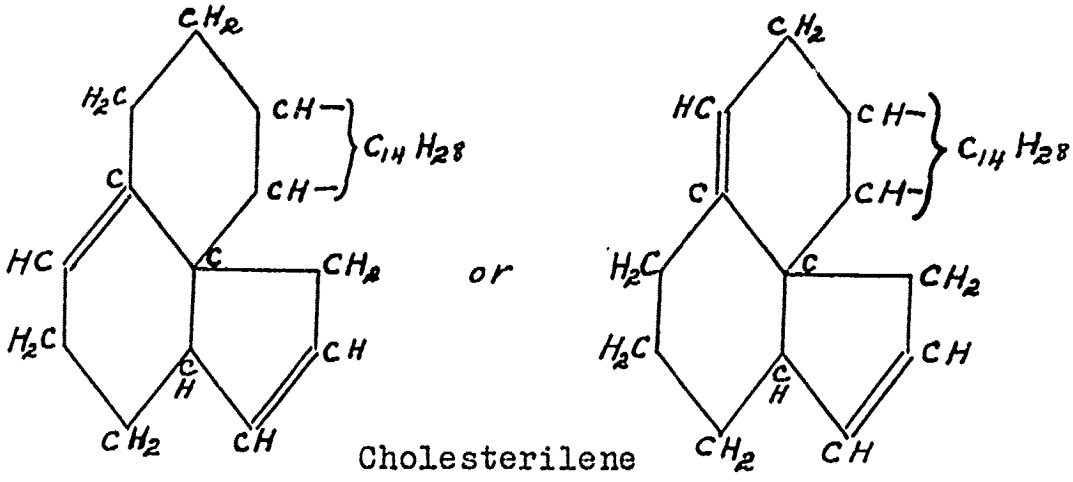
It is evident that one of the double bonds of cholesterilene occupies the same position as in pseudo-cholestene. That authors believe that the formation of cholestane and pseudo-cholestane by the catalytic hydrogenation of pseudo-cholestene can only be explained on the basis of these formulae:



Pseudo-cholestene

Assuming, as seems probable, that the double bond present in cholesterol at 6:7 does not change its

position, there are two possible structures for cholesterilene, either of which is in agreement with the production on hydrogenation of the stereoisomeric saturated hydrocarbons, cholestane and pseudo-cholestane.



Cholesterilene

On the basis of absorption spectra it is assumed that two of the three double bonds of ergosterol occupy the 1:2 (or 1:13) and the 6:7 positions. The presence of a double bond in the 1:2 (or 1:13) position is supported by the fact that ergosterol gives an immediate red color with trichloracetic acid and with chloral. The immediate production of color with these reagents was found by Rosenheim[26] to depend upon the presence of a double bond in the 1:2 (or 1:13) position.

In a study of the catalytic hydrogenation of ergosterol, Heilbron and Sexton[27] found a sharp break

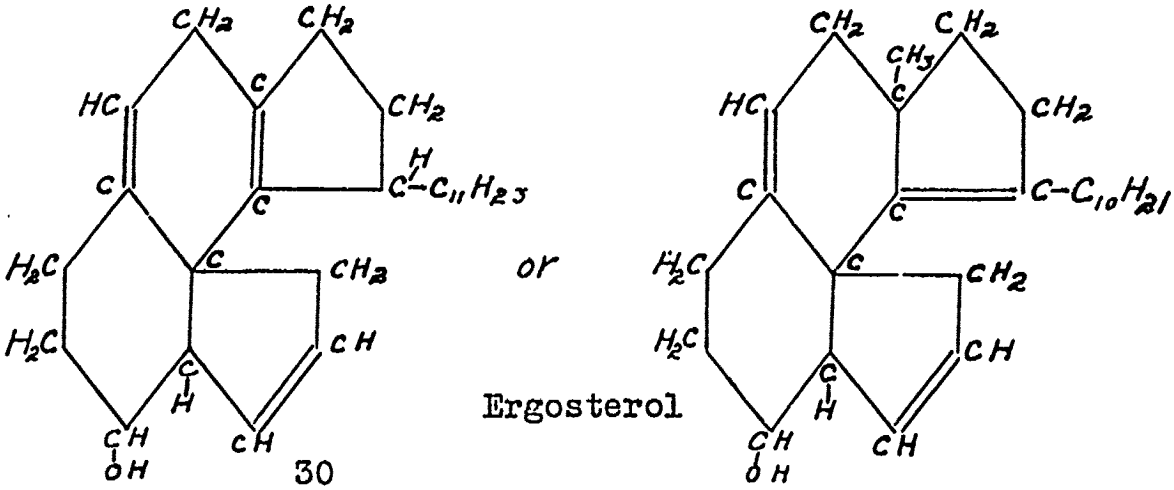
in the rate after the addition of two atoms of hydrogen in ether solution. The process may be arrested at this stage and dihydroergosterol isolated. The use of glacial acetic acid at a temperature of 70° C results in the formation of saturated allo-α-ergostanol acetate. Windaus and Linsert succeeded in adding six atoms of hydrogen to ergosterol in hot glacial acetic acid containing hydrochloric acid in the presence of platinum black.

Heilbron and Sexton do not believe it probable that the complete saturation of ergosterol under their conditions results in the shift of a double bond. They argue that of the three double bonds in ergosterol, the one which resists hydrogenation in ether or alcohol must be the one which is not present in cholesterilene, since no difficulty is experienced in fully hydrogenating cholesterilene in ether at room temperature.

Abietic acid requires the use of hot glacial acetic acid as solvent to bring about complete saturation with hydrogen. Because the resistant double bond in this compound joins two quaternary carbon atoms these authors suggest tentatively, by analogy, that in ergosterol the double bond which resists hydrogenation may be similarly placed. Such a position is to be found between carbon atoms 10 and 11, or according to

the work of Wieland and Vocke[28] which indicates a methyl group attached to carbon atom 11, the resistant double bond must be between carbon atoms 10 and 19. The presence of a double bond resistant to hydrogenation is also indicated by a color reaction investigated by Heilbron and Spring.[29]

Upon the basis of these findings, Heilbron and his associates propose this formula for ergosterol:

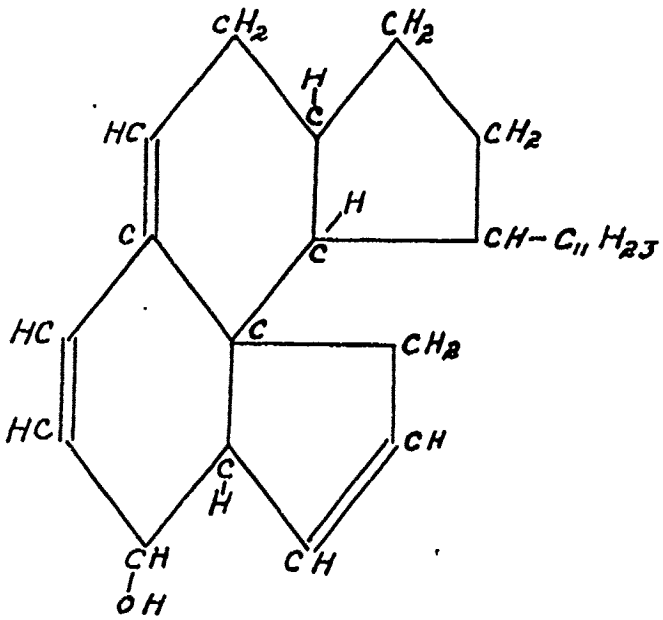


Ergosterol

Windaus[30] studied the position of the double bonds in ergosterol from the point of view of absorption spectra and the results of hydrogenation with sodium and ethyl alcohol. It was assumed that if several unsaturated isomeric hydrocarbons or alcohols show different ultra-violet absorption spectra, the compound which absorbs most strongly in the long wave region probably contains conjugated double bonds, and further, that the presence of conjugated double bonds is extremely probable in a compound which, in contrast

with its isomer, takes up two atoms of hydrogen when treated with sodium and alcohol.

Ergosterol is transformed to dihydroergosterol upon treatment with sodium and alcohol; none of the isomers investigated was hydrogenated by this treatment. Ergosterol shows its chief absorption at 280 mu, isoergosterol at 247 mu, and ergosterol D at 240 mu. Windaus therefore argues that ergosterol most probably contains a system of conjugated double bonds which, judging from the type of hydrogenated compounds formed, are in two different rings. He proposes this structure:



Ergosterol

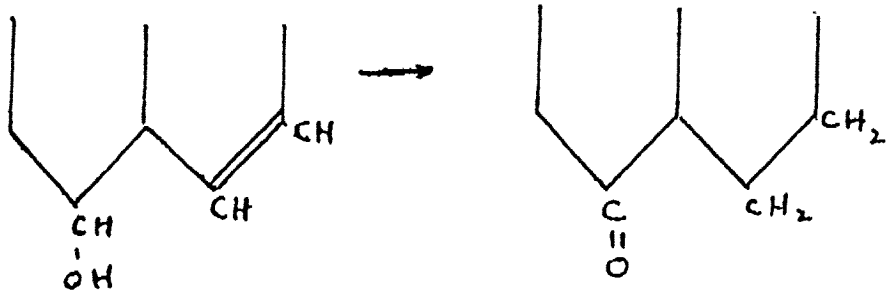
The Nature of Vitamin D

The nature of the change undergone by ergosterol when it is converted to vitamin D is not known. Of the different and conflicting opinions the two for which the most convincing evidence is offered are (1) that ergosterol is isomerized, and (2) that vitamin D is probably a ketone. It would be quite impossible to give all of the evidence for these opinions, but a few representative papers will be reviewed.

Windaus and his collaborators believe that vitamin D is an isomer of ergosterol. The fact that activation takes place in the absence of air would suggest isomerization or polymerization under the influence of ultra-violet light. Molecular weight determinations show that polymerization is not probable.

Windaus and Linsert[31] consider four possibilities for isomerism:

1. A transfer of two hydrogen atoms from the secondary alcohol group to a double bond. A change of this sort actually takes place when cholesterol or sitosterol is heated to 220° C with nickel.



The authors determined the alcohol group in ergosterol and in irradiated ergosterol and concluded that this group does not disappear, hence they regard this first possibility as untenable.

2. A double bond may form a ring linkage as a result of irradiation. According to the results of titration with perbenzoic acid and of catalytic hydrogenation, the number of double bonds in ergosterol does not appear to be altered by irradiation.

As isomerization possibilities there remain:

3. A rearrangement of the position of the double bonds.

4. A steric rearrangement at the secondary alcohol group, at the double bonds, or in some other place in the molecule.

Several of these changes may take place, either simultaneously or in succession, but in any case, the non-precipitatability with digitonin of the compound or compounds resulting from irradiation indicates a steric change at the secondary alcohol group.

Windaus also states that since irradiation isomerizes dehydroergosterol, it seems reasonable to assume a similar change for the closely related ergosterol. Later work has strengthened his conviction that vitamin D is an isomer of ergosterol containing

three double bonds and a secondary alcohol group.[32, 33]

Bills and his coworkers[34] are also of the opinion that vitamin D is an isomer of ergosterol. They state that the earliest photochemical decomposition product of vitamin D exhibits an absorption and an extinction coefficient which apparently identifies it with isoergosterol. As evidence of the importance of the alcohol group in activation Bills notes that although ergosteryl-isoergosteryl ether shows the absorption bands typical of ergosterol it can not be activated.[35]

Bills and Brickwedde[36] activated cholesterol at -183° C, the temperature of liquid oxygen. Most bimolecular chemical reactions, especially oxidations, are largely inhibited at very low temperatures, while the rates at which photomolecular changes take place decrease more slowly with decreasing temperature. The fact that activation is possible at -183° is regarded by these investigators as strong confirmation of the evidence of Windaus that activation is the result of isomerization.

On the basis of ultra-violet absorption spectra studies, Reerink and van Wijk[37] also conclude that vitamin D is an isomer of ergosterol.

On the other hand, Heilbron, Morton and Sexton[23] state, "It is also unlikely, in view of the total alteration of the type of selective (ultra-violet) absorption, that the reaction is due simply to stereochemical changes in the molecule, for cis- and trans-isomerides usually differ in extinction rather than in the general shape of the absorption curve."

The absorption spectrum of cholestenone[23] shows a shallow band at 312 mμ, probably a ketone band, and a band at 243 mμ which is strikingly similar to the well-defined band at 247 mμ of vitamin D. The change of absorption of the vitamin and of cholestenone on irradiation are similar in that the band at 243 mμ disappears rapidly, the ketone band less rapidly. It is a well established fact that cholesterol readily undergoes intramolecular rearrangement, the type of compound isolated depending on conditions. The fundamental reaction, however, would appear to be dehydrogenation leading to cholestenone formation, and it may well be that the conversion of ergosterol to the vitamin follows a somewhat similar course, giving rise to ergostatrienone, or a partially hydrogenated ketone derived from this. Although no band corresponding to the 312 mμ band of cholestenone has been observed in the spectrum of vitamin D preparations, it may possibly be

masked owing to the incomplete transformation of ergosterol.

Irradiated ergosterol gives a violet color with fuchsin and sulphuric acid, it also reduces ammoniacal silver nitrate. Non-irradiated ergosterol gives neither reaction.[38] Oils containing the antiricketic vitamin give a red color with aniline and hydrochloric acid.[39] Sexton investigated this reaction and found that all singly unsaturated ketones give the red color. Irradiated ergosterol gives a red color, while non-irradiated does not. Although none of the color reactions described is specific for vitamin D, they do suggest the presence in irradiated ergosterol of an aldehyde or ketone, which, of course, is not necessarily the vitamin.

Rosenheim and Adam[40] measured the monomolecular surface films on water of numerous sterols and sterol derivatives. The film of irradiated ergosterol strikingly resembles the films of certain ketone derivatives. The film of a very potent resin obtained by evaporating the filtrate in vacuo after precipitating unchanged ergosterol, in particular, resembles that of ketocholesterylene (cholestadienone, 8-one). The ultra-violet absorption of these two substances are very similar. Ketocholesterylene is antiricketically

inactive.

The evidence presented suggests the presence of ketone derivatives among the products of irradiation. Irradiation may induce intra- or inter-molecular dehydrogenation of the CH(OH) group, the double bond of the same or of a second molecule of ergosterol functioning as the hydrogen acceptor. This reaction would lead to the formation of ketones with two or three double bonds, of which the former is isomeric with ergosterol. The ketones possess the known chemical characteristics of vitamin D; as ketones they are unable to combine with digitonin, as enols they may form esters.

It must be remembered, however, that these highly absorptive unsaturated ketones may constitute the biologically inactive portion of irradiated ergosterol. Highly absorptive oxycholesterylene as well as several ketones derived from ergosterol have been found inactive. The ultra-violet absorption of oxycholesterylene may be due to a system of conjugated double bonds and a similar system may be responsible for the absorption bands of ergosterol.

The assumptions concerning the nature of vitamin D are largely based upon ultra-violet absorption spectra. Smakula[41] interpreted a study of differences

in the spectra of ergosterol and irradiated ergosterol to indicate that vitamin D is characterized by two bands at 293 and 262 mμ. Bourdillon, Fischmann, Jenkins and Webster[42] associate vitamin D with a single band of great intensity at 280 mμ. Reerink and van Wijk[37] calculate for vitamin D a single broad band with a maximum at 280 mμ. The band at 248 mμ often associated with vitamin D is evidently due to a degradation product.

Jendrassik and Kemenyffi[43] report a highly active irradiated ergosterol which, after removal of the unchanged ergosterol showed only general absorption in the ultra-violet.

Windaus[44] states that "We must speak with the greatest caution concerning the significance of the (ultra-violet) absorption spectrum in characterizing vitamin D. We have not yet examined a potent preparation which did not absorb between 260 and 290 mμ. On the other hand, we have repeatedly had irradiated ergosterol which in spite of intense absorption at 280 mμ was only weakly antiricketic."

THE RELATION OF INFRA-RED ABSORPTION TO CHEMICAL CONSTITUTION

Definite infra-red absorption bands are manifestations of definite energy relationships. Drude,[45] from a conception of vibrating charged particles, worked out a mathematical relationship between given wave lengths in the ultra-violet and corresponding wave lengths in the infra-red spectrum. Since the frequency of electro-magnetic oscillations of infra-red radiation is slow compared with the rate of motion of electrons in atomic orbits, emission and absorption can occur only for charged masses moving at a velocity much less than that of the electrons, i. e., for ions, atoms or molecules. The infra-red absorption spectrum, therefore, is characteristic of the structural groups of the molecule.

Bjerrum[46] suggested that in addition to the frequencies due to the vibrations of charged ions and atoms, a molecule possesses a frequency due to its rotation, and that this rotational frequency accounts for the second, and apparently quite distinct, series of bands in the far infra-red spectrum.

The infra-red spectrum thus falls naturally into two regions, that in the near infra-red, the "vibration" or "rotation-vibration" spectrum, and that in the far in-

fra-red, the "pure rotation" spectrum. Lecomte[47] states that the bands of the vibration spectrum characteristic of structure are found at wave lengths rarely exceeding 14 μ, while those of the rotation spectrum do not occur below 8 to 10 μ, rarely below 14 μ.

For purposes of classification the near infra-red is usually considered as the region extending from .8 μ, the extreme of the visible red, to 23 μ in the infra-red; this region can be explored by the use of suitable prisms. The far infra-red extends from 23 μ to about 300 μ.

THE PROBLEM

The molecule of ergosterol is large and complex, but it contains only carbon, hydrogen and oxygen and these in relatively few different types of linkage. The complexity of infra-red absorption spectra increases with the number of distinct atoms or groupings in the absorbing molecule rather than with the general complexity or size of the molecule itself. The literature contains data as to the positions of bands identified with the hydroxyl group, the ketone group and with isomeric relationships. An investigation of the spectra of ergosterol and irradiated ergosterol and of isomeric and ketonic derivatives could reasonably be expected to

throw some light on the nature of vitamin D.

The spectra of a number of derivatives which have been prepared for this study will be presented in a later paper. This preliminary discussion will be confined to the infra-red spectra between .8 μ and 3 μ of ergosterol and irradiated ergosterol.

EXPERIMENTAL

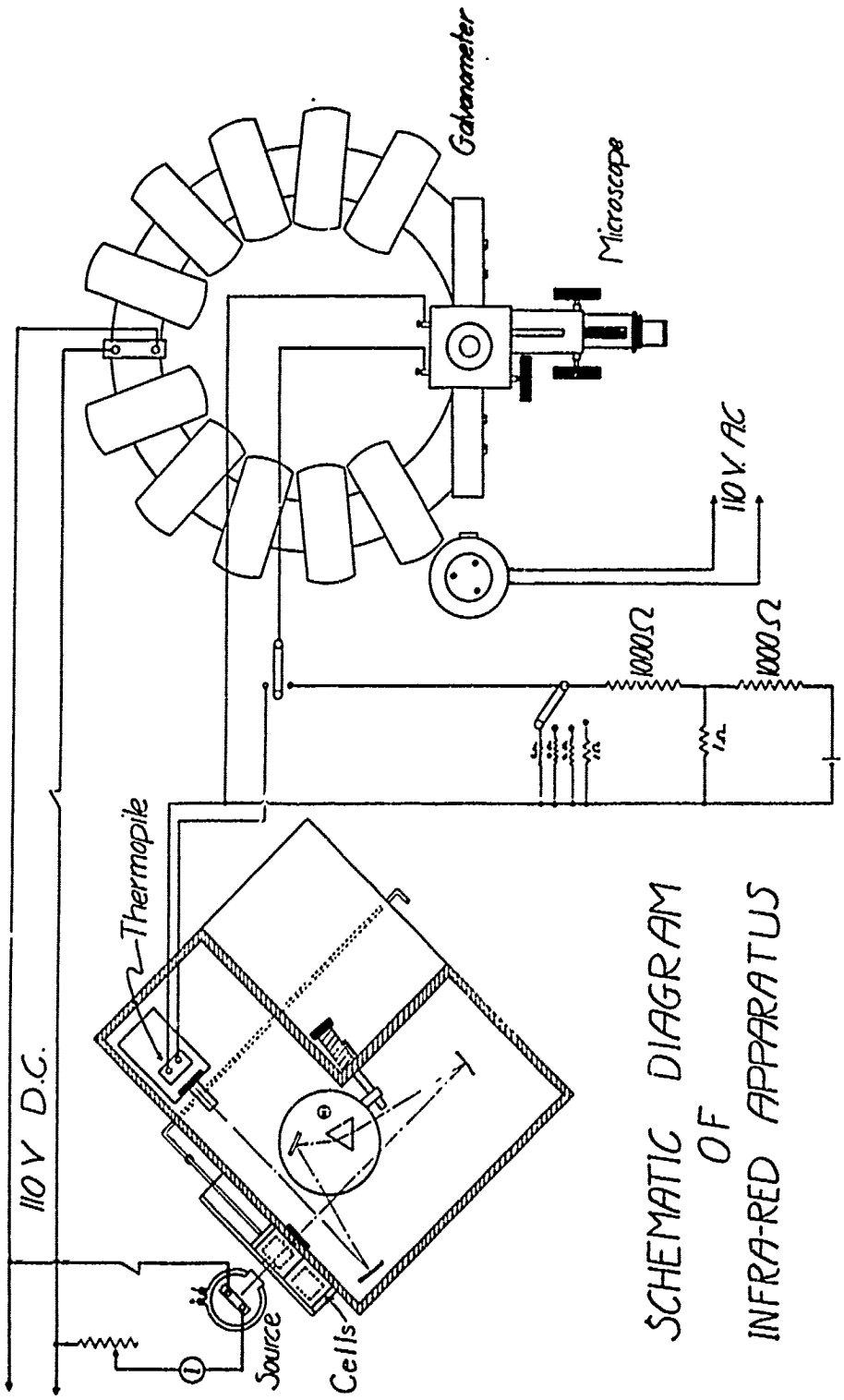
Apparatus

Fig. 1 shows a diagramatic representation of the infra-red apparatus. With the exception of the spectrometer and the thermopile, this apparatus was built in the Basic Science Research Laboratory.[48]

The most satisfactory source of infra-red radiation was found to be a rod of carborundum and graphite, the "globar" rod, heated by the passage of a current. The rod, 5/16" in diameter, is ground to a diameter of 3/16" at the middle, in order to concentrate the heat at this point, which is the source of the rays. It is important that the current through the rod be constant. The current is controlled by a large carbon pile rheostat connected in series and is maintained at 8 amperes. An ammeter is included in the circuit. The rod is enclosed in a housing to permit proper contact; this housing is water cooled to prevent heat radiation. The spectrum of the globar rod shows the absence of ultraviolet radiation so that activation of ergosterol by exposure to this radiation is excluded.

The absorption cells, 2, 5 and 10 cm long, are brass tubes with quartz windows. Two cells fit precisely into a slide which moves the cells in and out of the

path of the rays. Stops on the slide make possible the accurate duplication of the position. This is essential since internal reflection from the walls of the cell varies with position.



SCHEMATIC DIAGRAM OF INFRA-RED APPARATUS

The Hilger spectrometer is equipped with a 60° quartz prism giving a wave length range of 0.5μ to 3.5μ. This prism can be replaced by a fluorite prism, wave length range 0.38μ to 9.0μ; by rocksalt, 0.38μ to 17μ; and by sylvine, 0.38μ to 21.0μ. In selecting prisms attention must be paid to dispersion as well as to transparency.

The elements of the spectrometer are an adjustable entrance slit, a concave mirror, the quartz prism, a plane mirror, a concave mirror, and an adjustable exit slit behind which the thermopile is mounted. A slit width of .02 inches was used for this investigation.

The slits and the concave mirrors are rigidly fixed in a cast iron base which is mounted on a brass table provided with leveling screws. The prism and plane mirror are mounted on a table which is rotated by a fine steel screw, the point of which pushes against a projecting arm on the prism table. To the screw is attached a drum, calibrated to .01μ, on which the wavelength of the line under observation is read directly.

Rays entering the first slit are collimated by the first concave mirror and pass through the quartz prism to the plane mirror. From this they are reflected

to the second concave mirror which gives an image of the spectrum on the second slit. Rays of the wave length read on the drum pass through the slit to the thermopile.

The Hilger linear thermopile has a sensitive area 10 mm by .5 mm. The entire thermopile case is enclosed in a nickel case to protect it from external radiation. The receiving plates of the thermopile are of silver foil smoked black to insure heat absorption. The couple is of Hutchins alloy (bismuth-tin and bismuth-antimony.) The cold junction is attached to silver wings bent to form an almost complete envelope around the junction. The thermopile is mounted in a brass case blackened on the inside to prevent reflection and to minimize the effect of external temperature changes.

The galvonometer was designed and built in the Basic Science Research Laboratory. It is a modification of the Einthoven type in that the silvered quartz fiber which is suspended in the strong magnetic field moves freely at the bottom, electrical contact being made by means of a strip of gold leaf 1 mm wide. This fiber is mounted in an airtight housing to protect it from air currents.

The motion of the fiber is observed through a

telescope which has a 125 division scale mounted at the focus of the eyepiece. A plane mirror mounted at an angle of 45° to the axis of the microscope translates the forward and back motion to a sidewise motion which can be read from the eyepiece. Fine adjustments permit the fiber to be moved into any position in the field.

Could all other factors be disregarded, the condition for maximum efficiency would require that the galvonometer resistance equal that of the thermopile. This is not, however, a practical condition since the thermopile resistance of 10 ohms would require a very heavy fiber. The most favorable fiber resistance for the apparatus in its present form has been found to be 100 ohms, which gives a sensitivity of 10^{-11} amperes a division.

Because of the diamagnetic property of quartz the fiber tends to move out of the field even when no current is flowing. At an equilibrium point the force due to the weight of the fiber equals that due to its diamagnetic property. As the fiber is moved farther out into the field, a point is reached where the diamagnetic force is not sufficient to hold the weight of the fiber in position, and it swings to the opposite side of the field. Just before this point is reached

the galvonometer is at its maximum sensitivity. Ordinarily it was not necessary to operate at maximum sensitivity because sensitivities of 10^{-9} amperes a division gave good readings in the range investigated.

This type of galvonometer is relatively insensitive to mechanical and electrical disturbances in its vicinity. Since the smallest deviation which can be read with accuracy is 1 division on the eyepiece scale, the larger the reading the more accurate the result. However, the larger reading requires operation at higher sensitivity and hence under conditions of greater instability. In general, readings of from 30 to 50 divisions were used. Such readings can be obtained with fair stability, but with a possible error of 2 to 3% on a reading. For this reason it is evident that two curves for the same compound may not check exactly, although an average of many curves may be expected to give an accurate result.

Procedure

The transmission at each wave length of the substance under investigation is obtained as follows:

The wave length drum of the spectrometer is set at the desired wave length.

With the infra-red rays cut off, the galvonometer

fiber is adjusted to zero on the eyepiece scale.

The cell containing the solution is placed in the path of the rays entering the collimator slit, and the galvonometer deflection is recorded.

The cell containing the solvent is brought into the path of the rays and the galvonometer deflection read.

The ratio of the reading for the solution and the reading for the solvent gives the percent of energy transmitted by the solute at that wave length.

The instrument is calibrated against the well-established bands of chloroform.

Ergosterol

All of the ergosterol used was recrystallized from a mixture of alcohol and benzene (3:1) until a specific rotation of -130° to -132° was attained.[49] Bills states that "ergosterol of exceptional purity" shows $[\alpha]_D^{20}$ -132°. The ergosterol used in earlier work had been purified by conversion to the acetate, but recrystallization from the alcohol-benzene mixture gives a compound as pure as that obtained by the more laborious method.

Although natural isomers of different rotation exist, all of the ergosterol extracted from yeast in

the laboratory and all of the samples purchased showed at least α_D^{20} —130° after several recrystallizations.

Irradiation

The purified ergosterol was dissolved in ether, 5 mg to 1 cc, and irradiated in sealed quartz tubes at a distance of 12 cm from a mercury arc screened by a filter which cut off sharply at $\lambda = 2960$. The solvent was evaporated under diminished pressure. Unchanged ergosterol was estimated by precipitation with digitonin.

Unfortunately, the periods of irradiation recorded are only approximate. The original plan was to prepare different series of the same biological potency, that is, with approximately the same amount of ergosterol converted. The period of irradiation was not timed with great accuracy. After the amounts of unchanged ergosterol had been determined, it was noticed that logarithms of the concentration of unchanged ergosterol in the series irradiated from 1 to 5 hours, plotted against time, fell close to a straight line. A monomolecular reaction requires that $\frac{1}{t} \cdot \log_e \frac{a}{a-x} = k$. The values of k determined from the percentages of unchanged ergosterol vary, but still suggest a monomolecular reaction. It is probable that slight differences in the times of irradiation account for the variation.

A study of the ultra-violet absorption spectra of ergosterol irradiated with light of wave length 254 mμ and with light of wave length longer than 275 mμ led Reerink and van Wijk[37] to conclude that the effects of long and short wave irradiation were quite different:-

"In the case of long wave irradiation, the reaction proves to be comparatively simple in so far as only one product is directly formed from ergosterol. This product itself, which appears to be vitamin D, is after longer irradiation gradually transformed into a new and much more stable product. As the rate of destruction of the first product is rather small, it is possible to convert about 60% of the ergosterol into vitamin D before the secondary reaction becomes important.

The short wave irradiation causes changes of a more complex character. In this case there are two reaction products present during the first period of irradiation. It is probable that one of these products, which is very quickly destroyed on prolonged irradiation, and thus does not accumulate to an appreciable amount, is identical with vitamin D."

The infra-red absorption curves of ergosterol irradiated with light of wave lengths longer than 2960 Å may be assumed to represent the absorption of ergosterol and of vitamin D only.

Ergosterol	$[\alpha]_D^{20}$	% Changed By Irradiation λ= 2960 A.					
		1	2	3	4	5	6 (Hours)
I	129.8	----	----	----	----	----	65.5
II	131.2	----	----	----	----	----	56.35
III	131.7	30.15	36.65	47.7	53.0	----	62.4
IV	131.6*	30.3	38.5	46.4	48.5	54.0	
V	131.6	34.0	41.2	42.4	46.0	48.5	
VI	131.6	29.5	38.0	45.1	48.0	53.0	
VII	131.6	27.1	34.2	42.3	49.0	52.3	
VIII	131.6	26.2	37.0	44.0	47.0	53.0	

*IV - VIII represent the same sample of purified ergosterol.

Potency

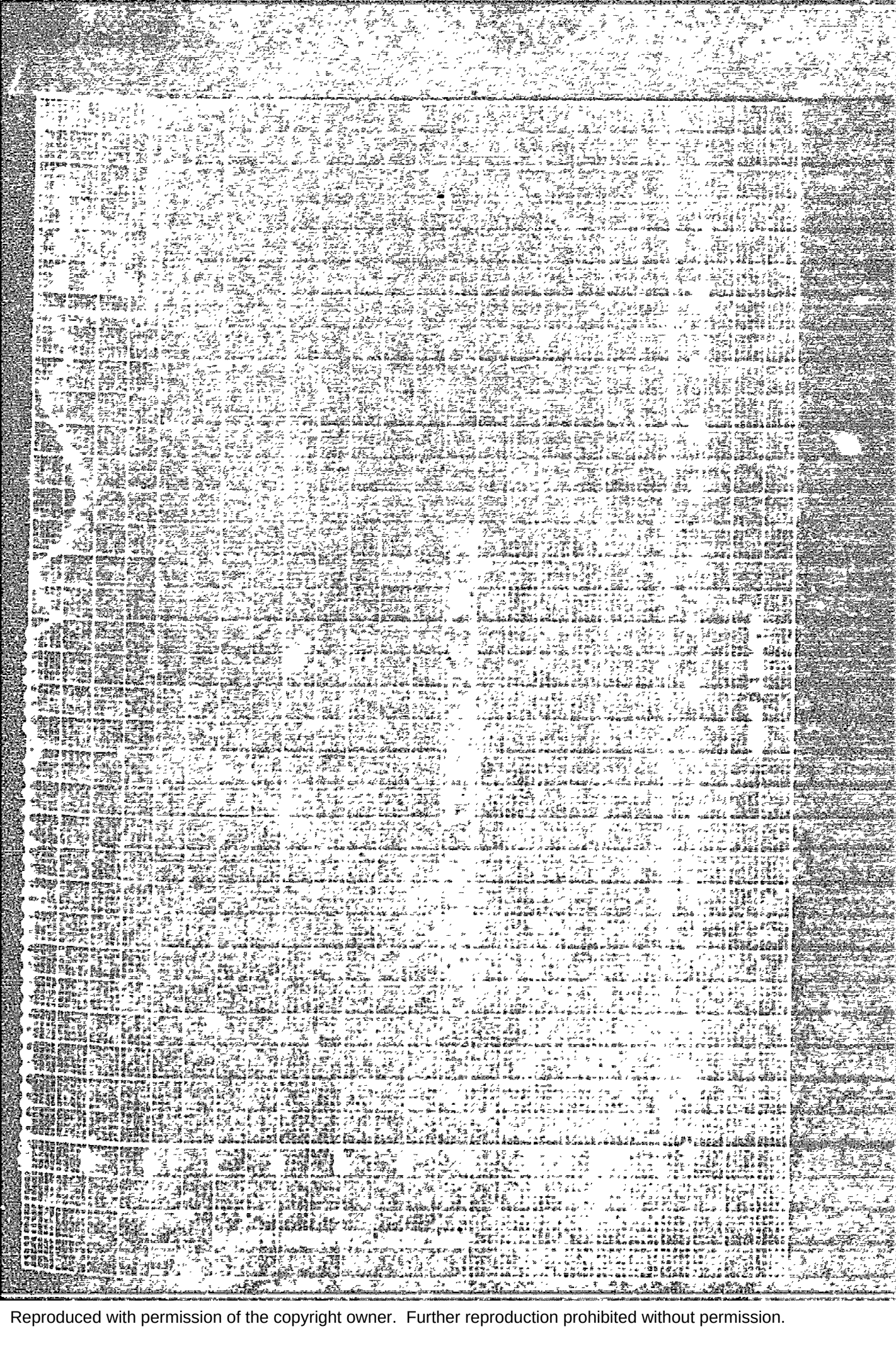
Ergosterol irradiated with filtered ultra-violet light λ = 2960 is very potent. X-ray pictures show that .000025 mg a day of preparations containing approximately 50% of unchanged ergosterol is curative for a rickets rat fed the McCollum diet. Smaller doses have not been tested on a large number of animals.

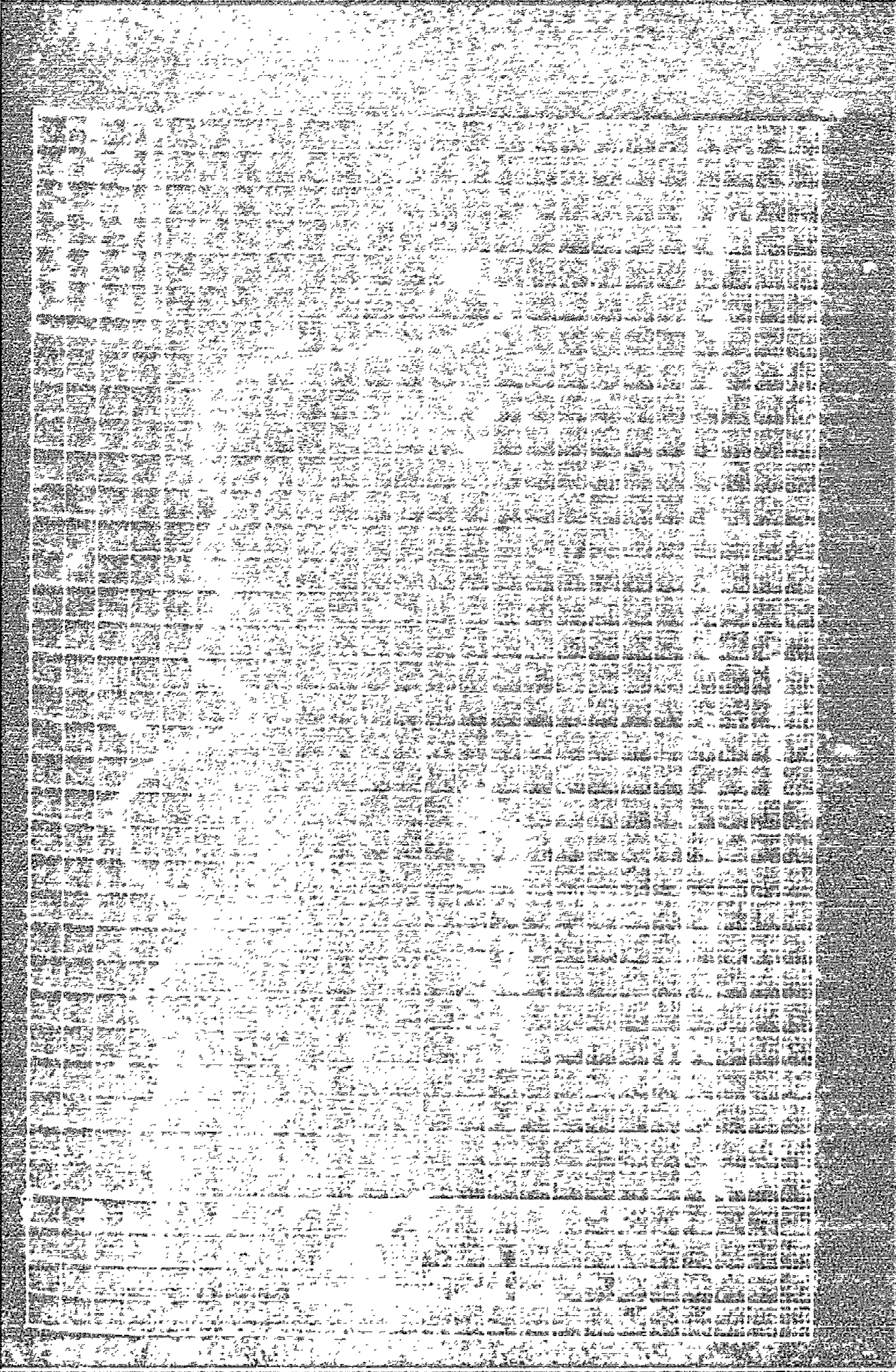
Solvents

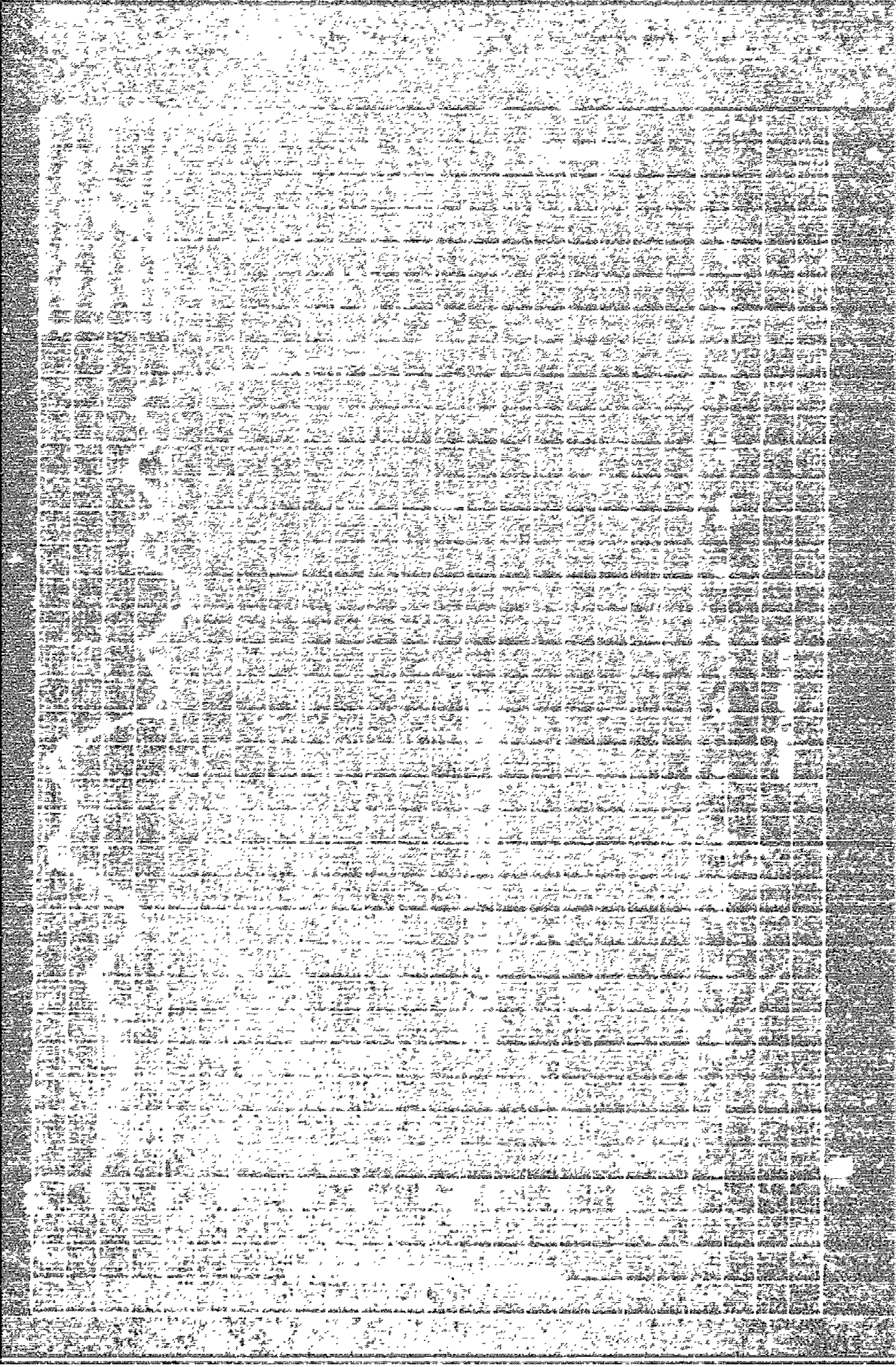
The selection of a suitable solvent for the solutions to be examined in the infra-red region proved difficult. Ether, alcohols, benzene, hexane and cyclohexane do not decompose ergosterol, but they all absorb strongly at critical points or they become opaque below 3 μ. A solvent which does not contain the C-H bond is required. Both carbon disulphide and carbon tetrachloride were used, but carbon disulphide is less transparent so that only thin layers of solution could be examined and many of the ergosterol bands did not show. Carbon tetrachloride is excellent from the stand point of transmission, but solutions of ergosterol in this solvent become yellow within sixteen to twenty hours. Solvents are still being investigated, but with proper precautions carbon tetrachloride is satisfactory, and was used for all of the curves presented in this paper.

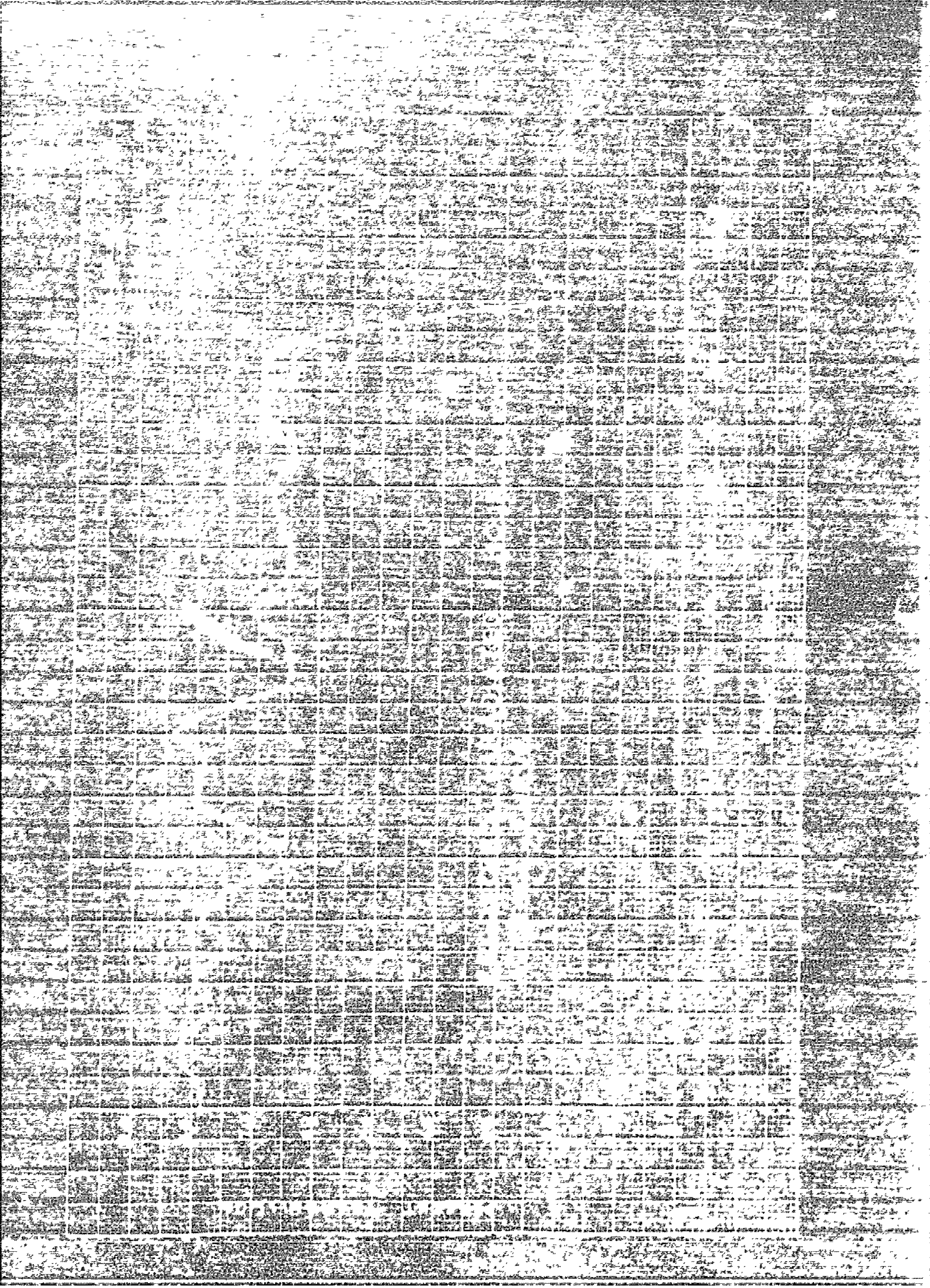
Most of the carbon tetrachloride used was purified and redistilled. After absorption curves of purified and "C. P." carbon tetrachloride had been found to check, and no difference in the behavior of solutions of ergosterol in either one could be detected, "C. P." solvent was used. It was tested for free chlorine and for chloroform before each solution was made, and fresh solutions were made for each curve. During the first year of the investigation the solutions were again tested after the curve was made, later only occasional samples were tested.

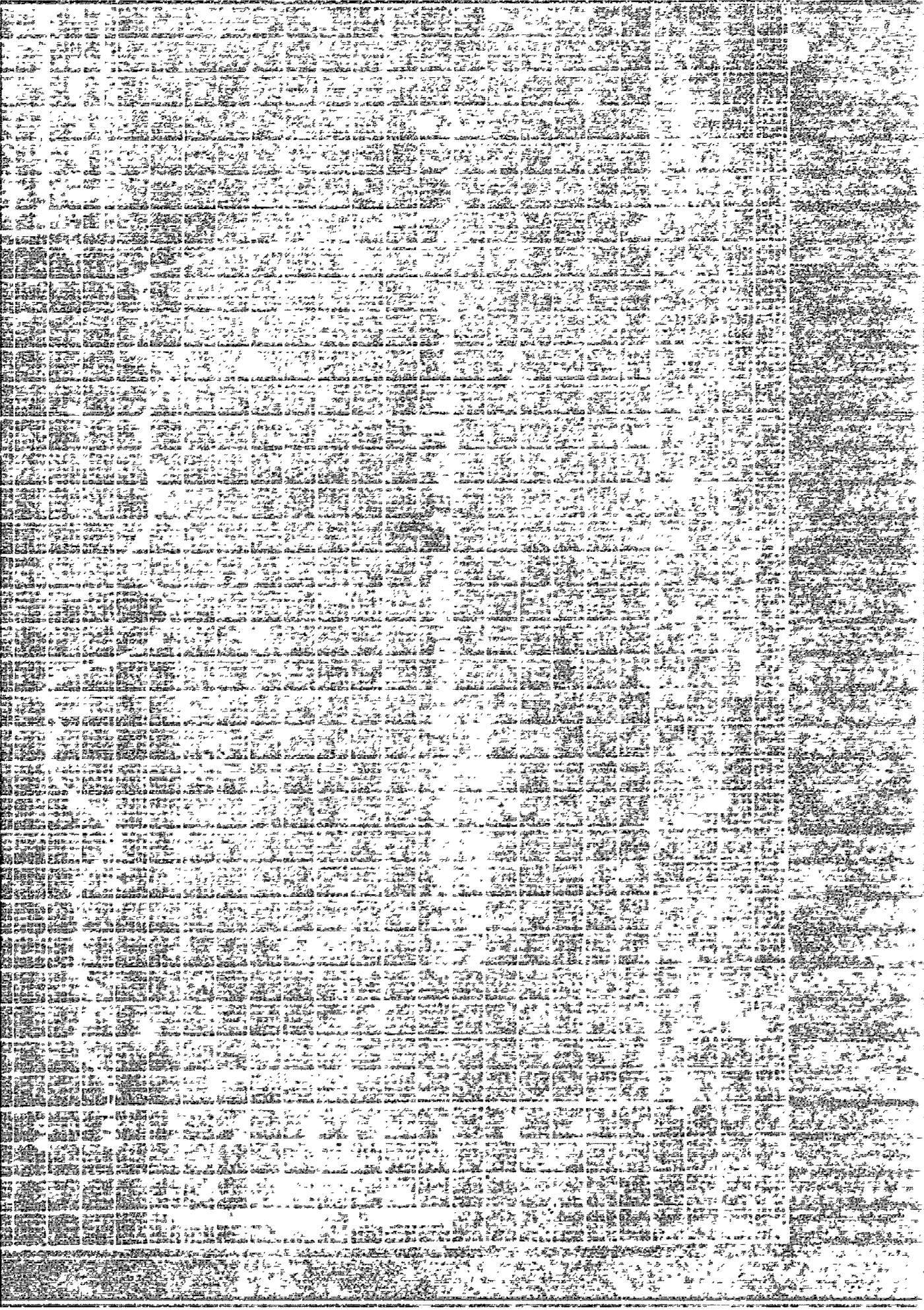
The fact that the different curves for ergosterol and for ergosterol irradiated with wave length 2960 A do not vary greatly may be taken as evidence that appreciable decomposition does not occur during the five or six hours required for the readings. The curves of ergosterol exposed to the unfiltered radiations of a mercury arc lamp can not be duplicated, probably because of the presence of unstable products of irradiation.

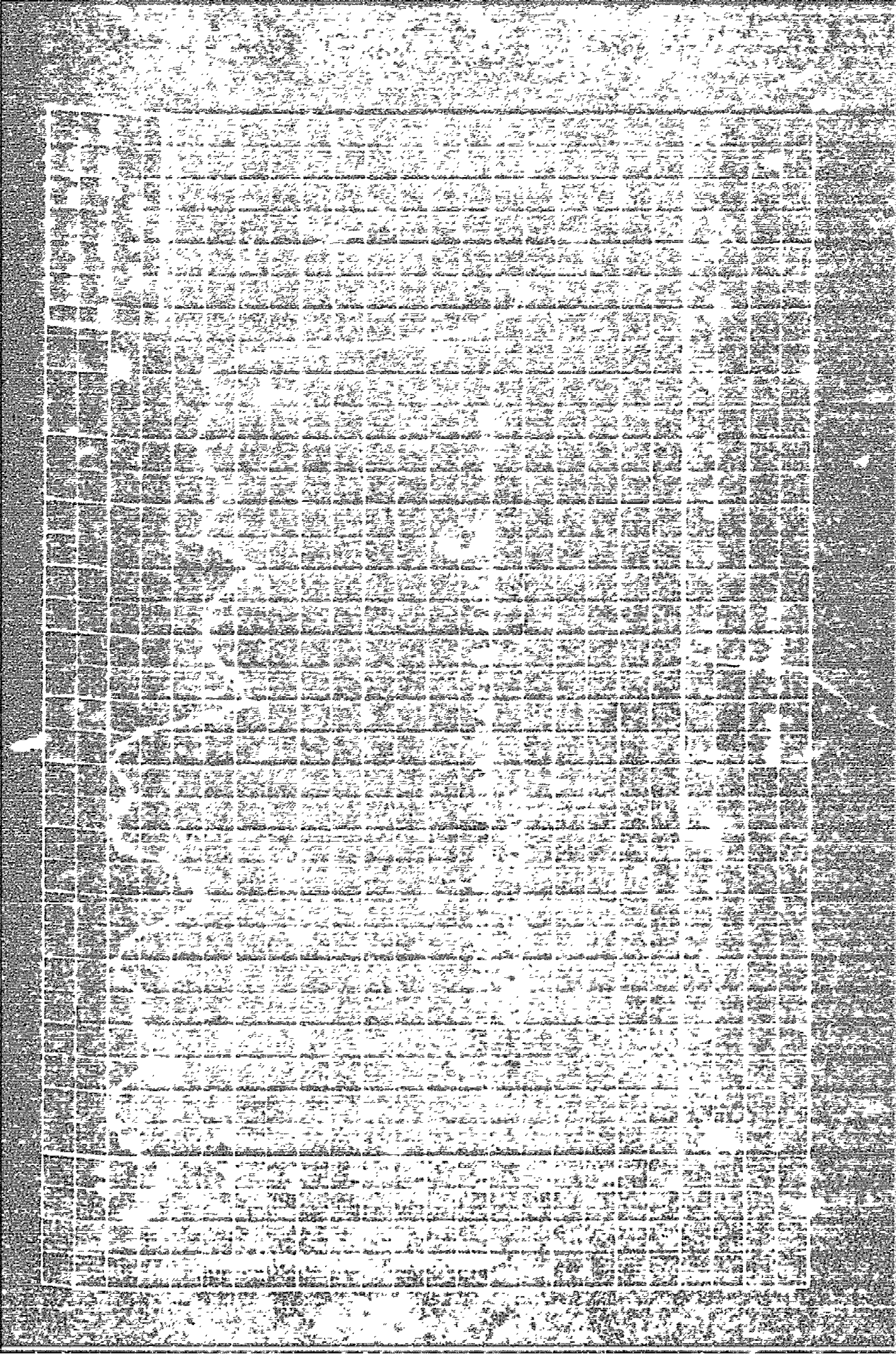


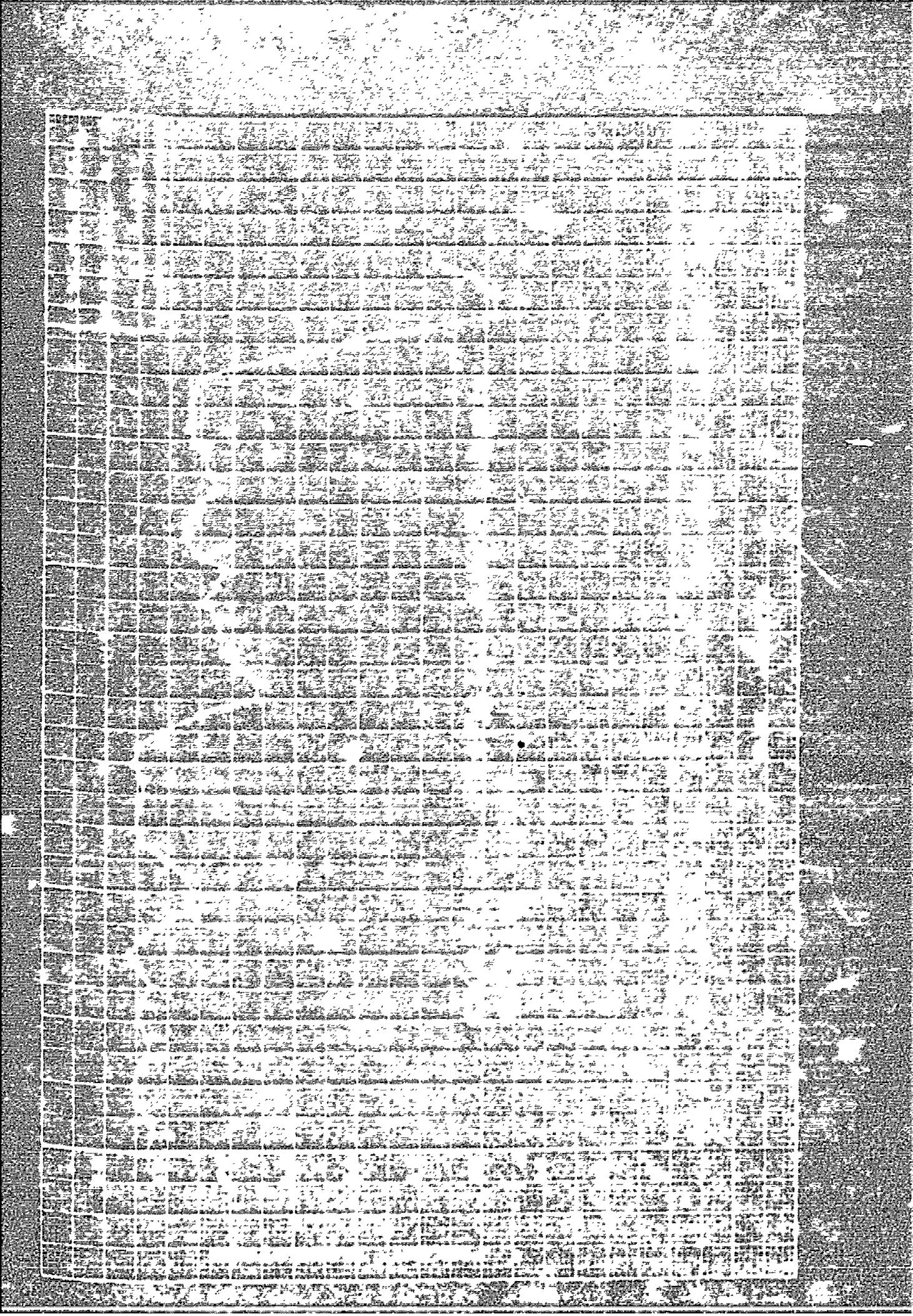


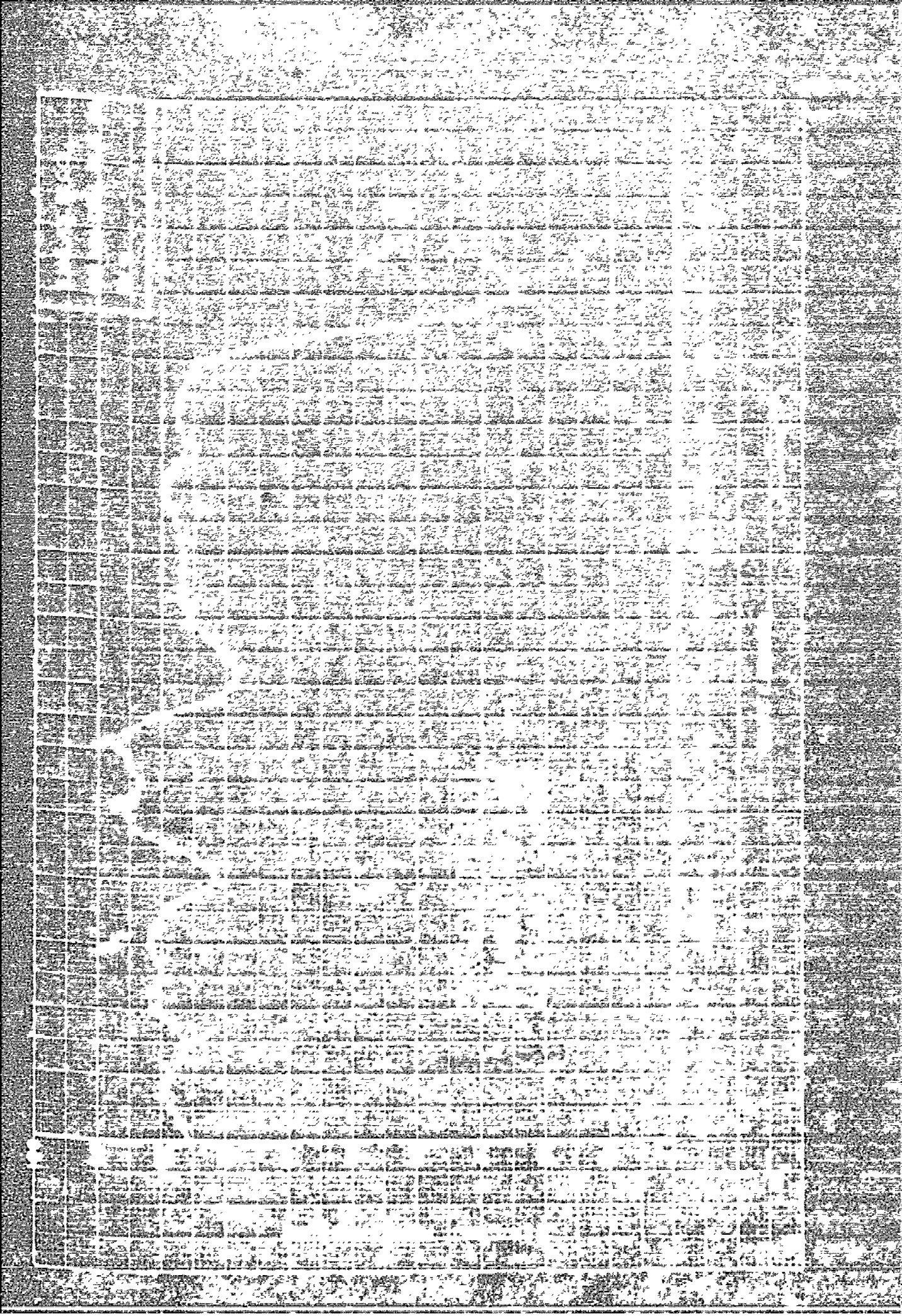


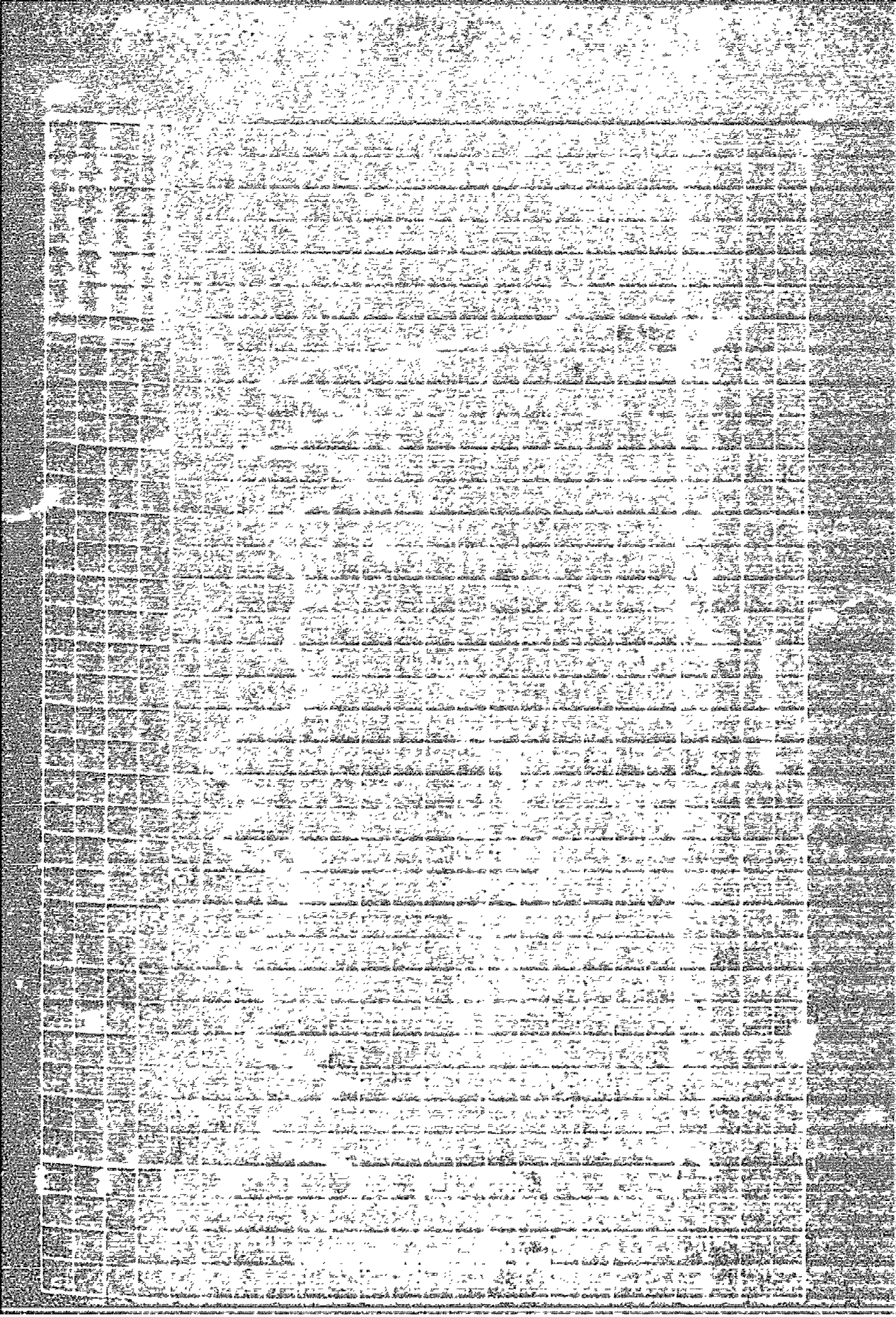


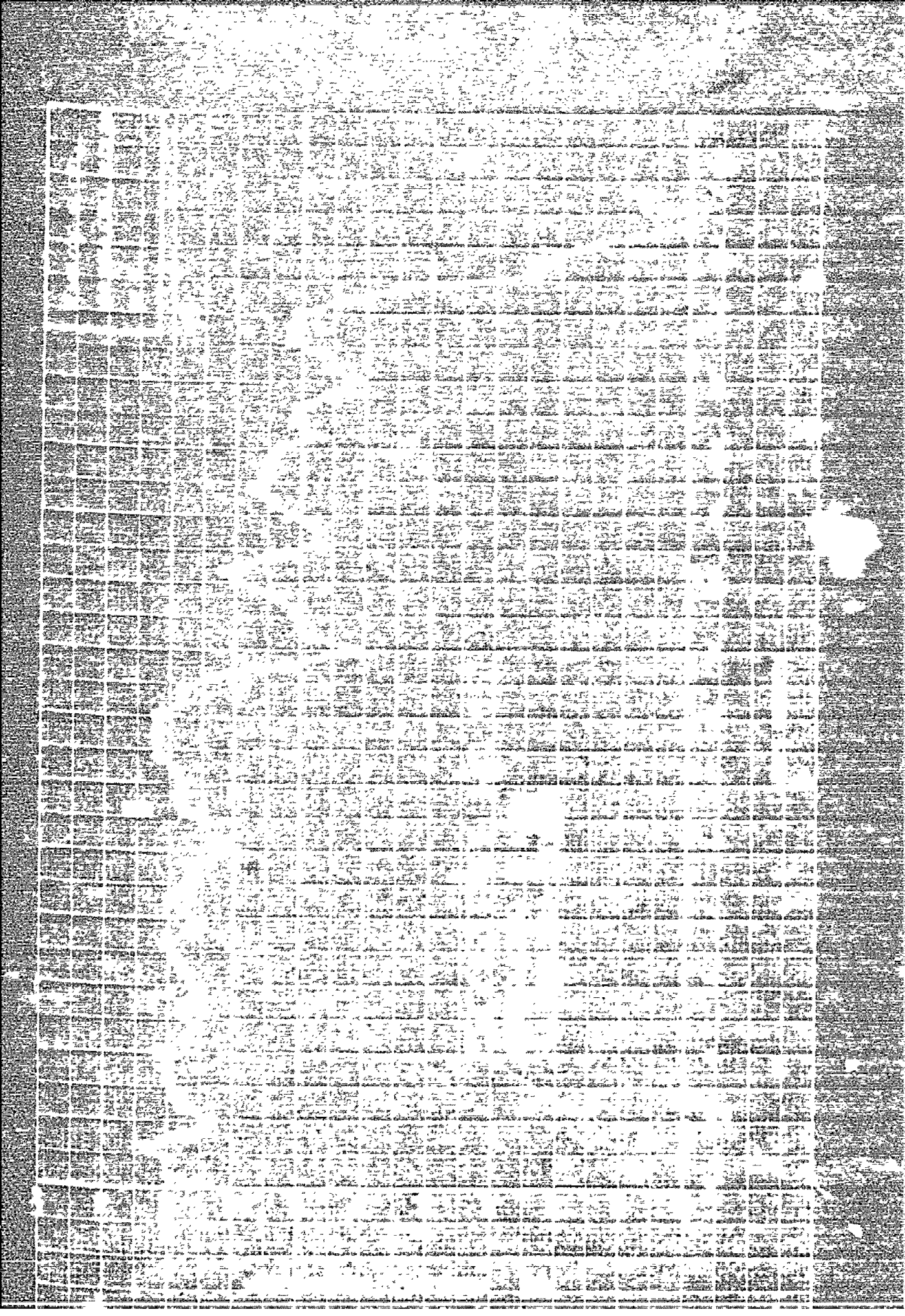


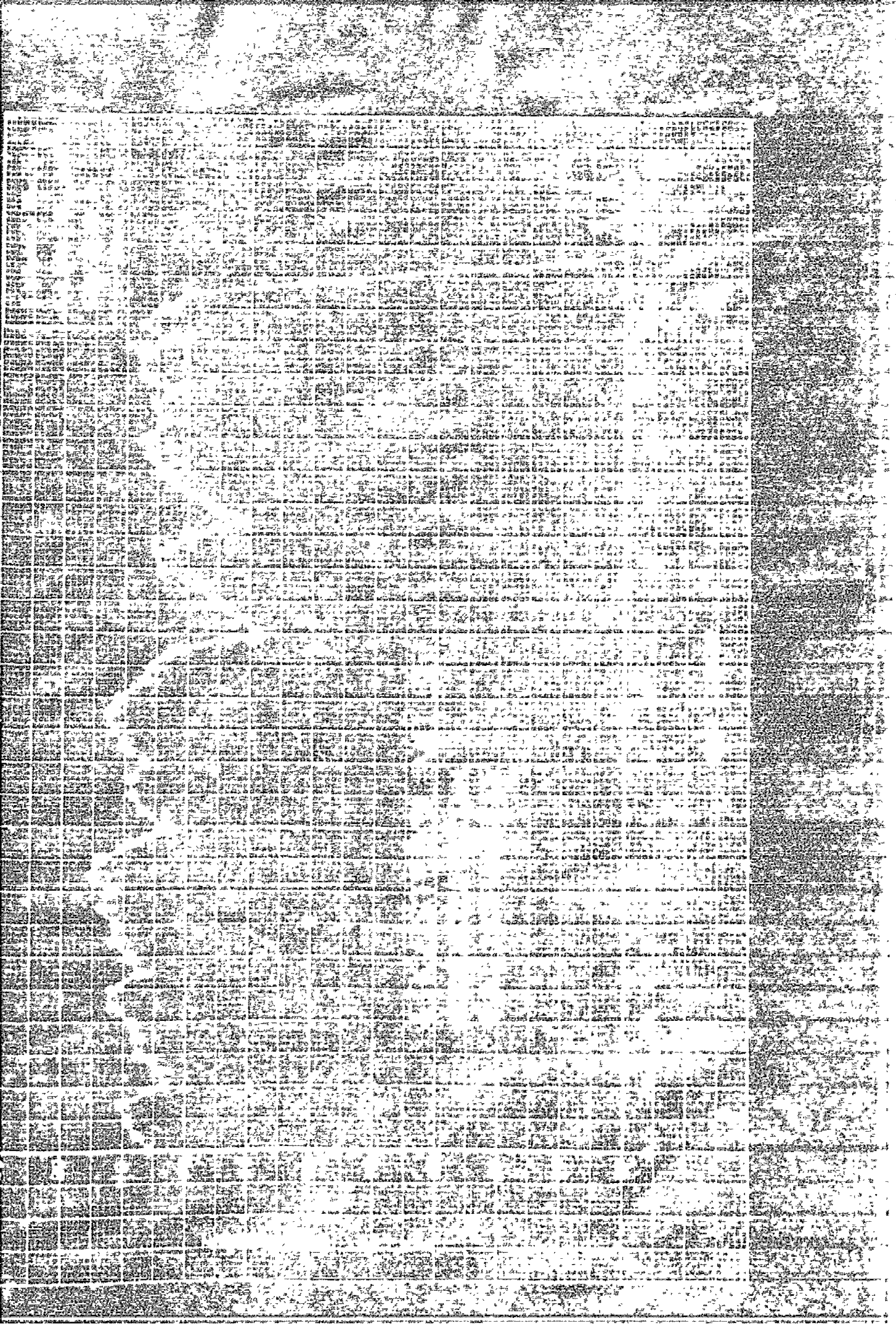


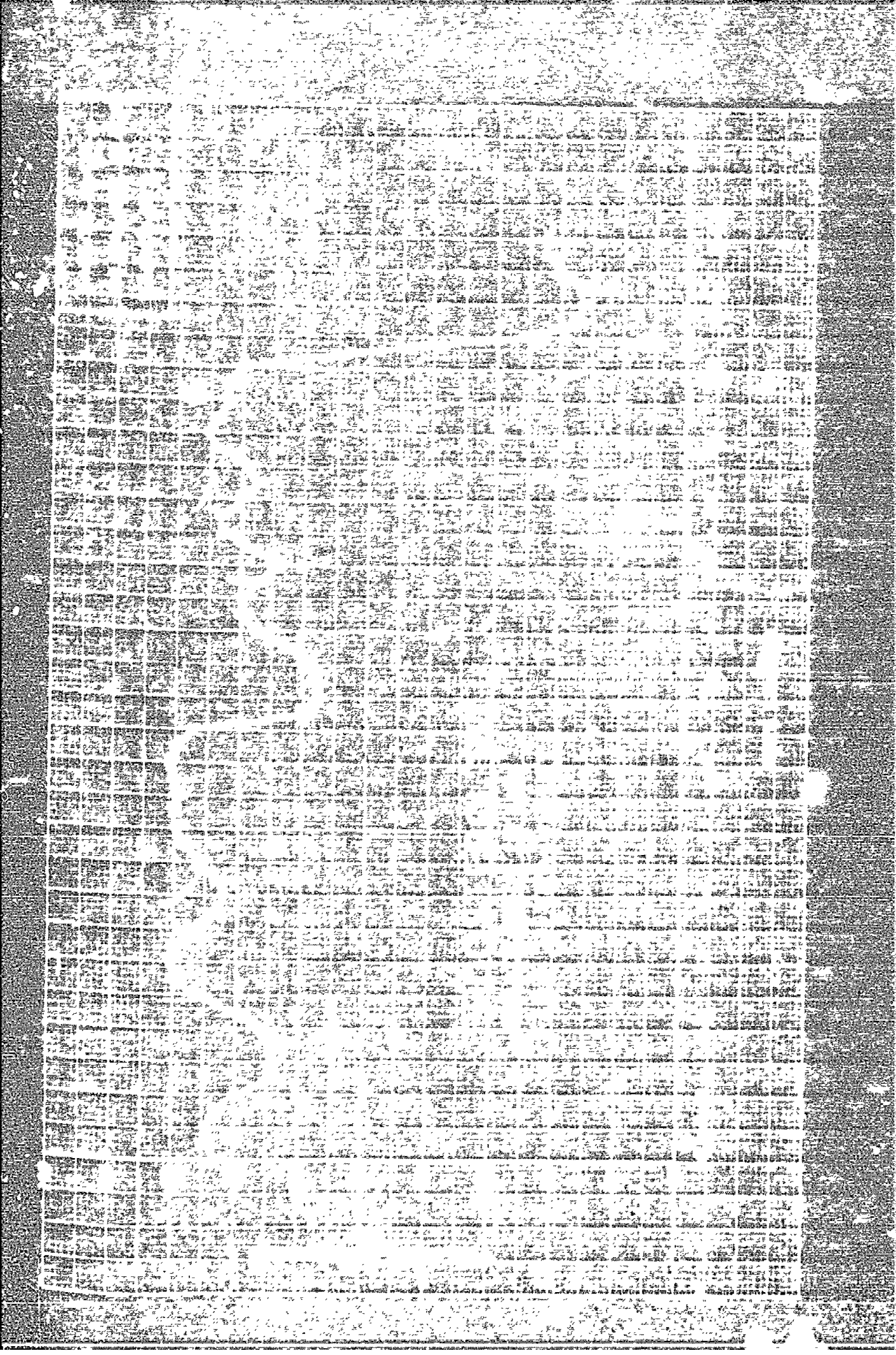


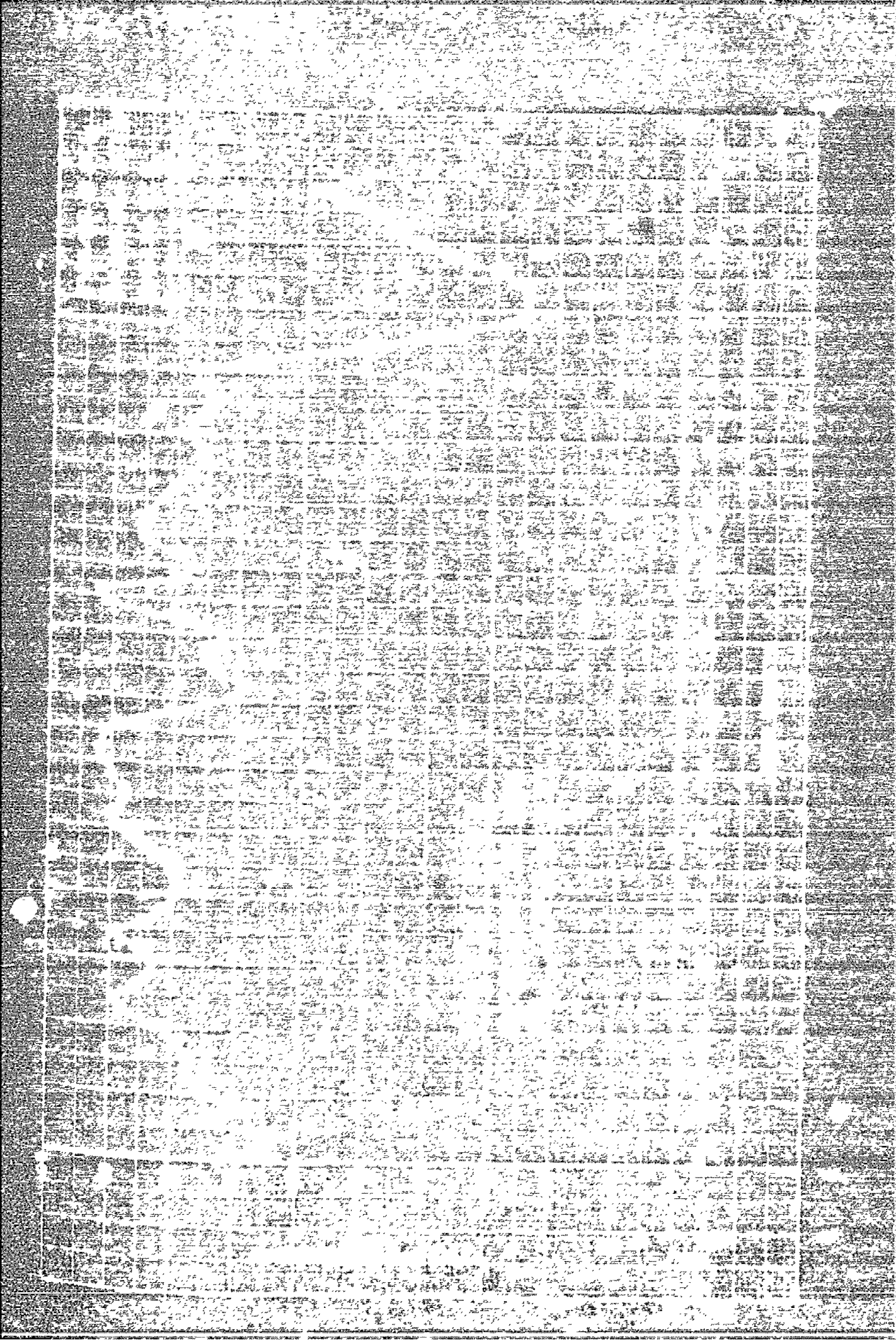


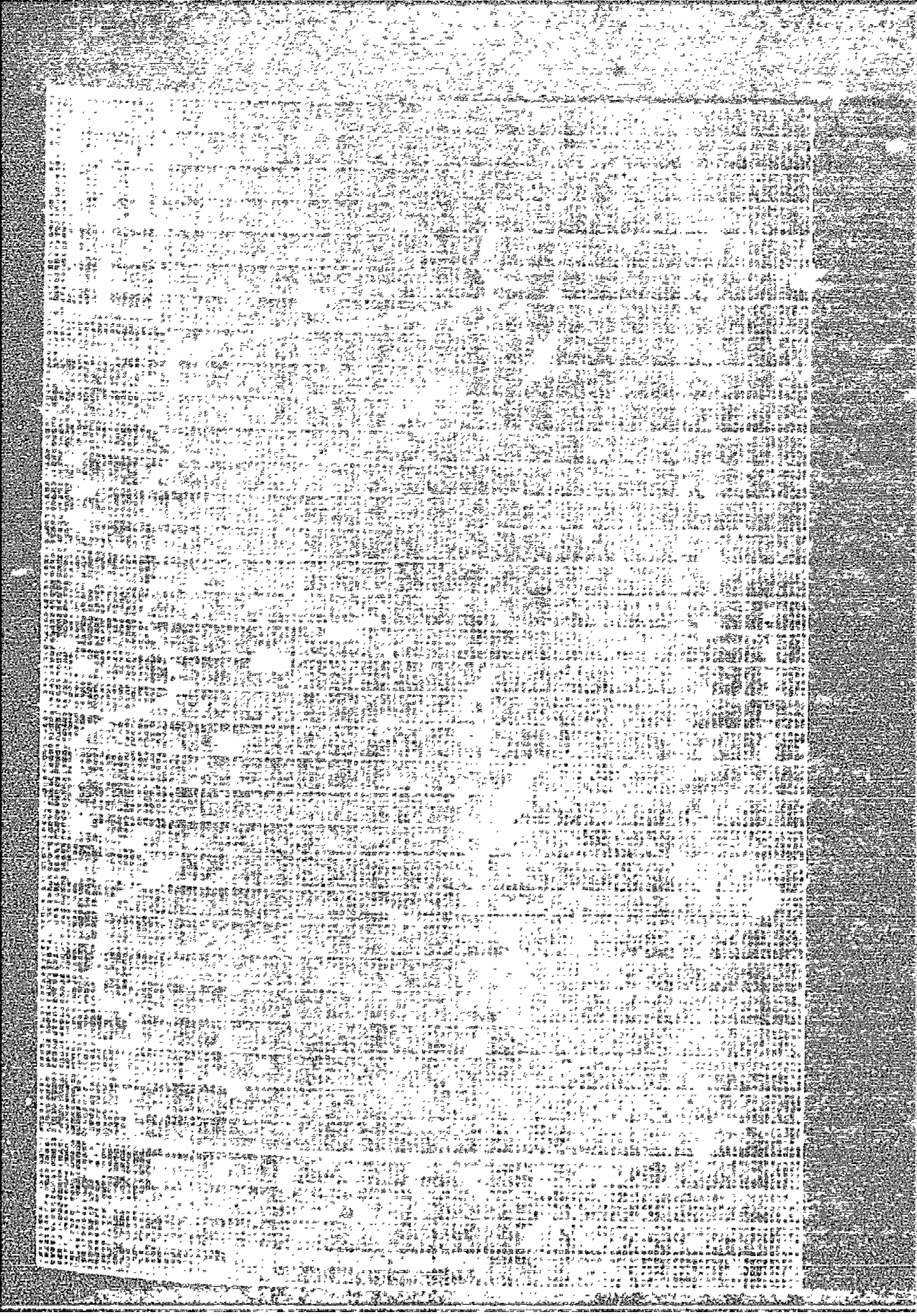


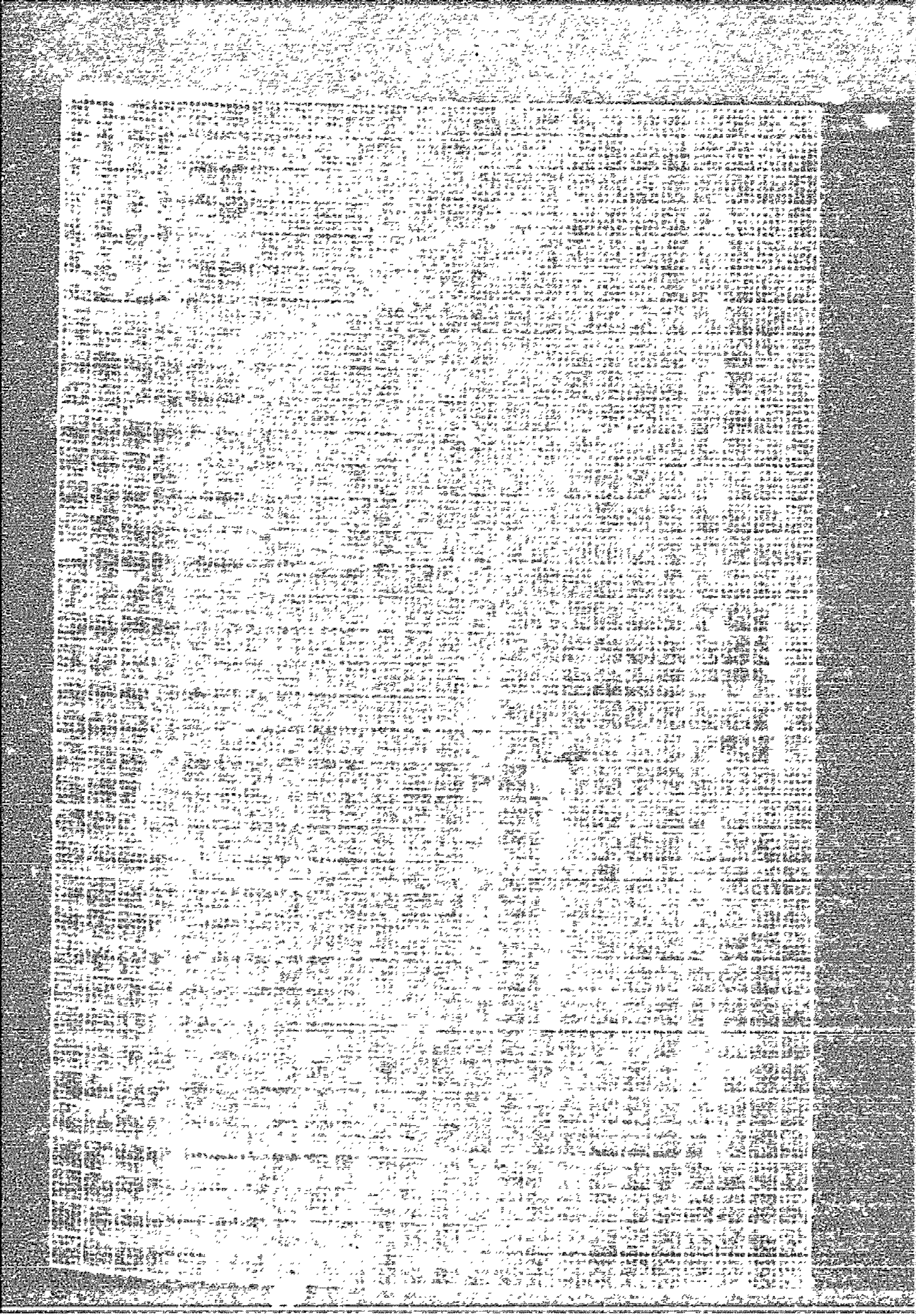


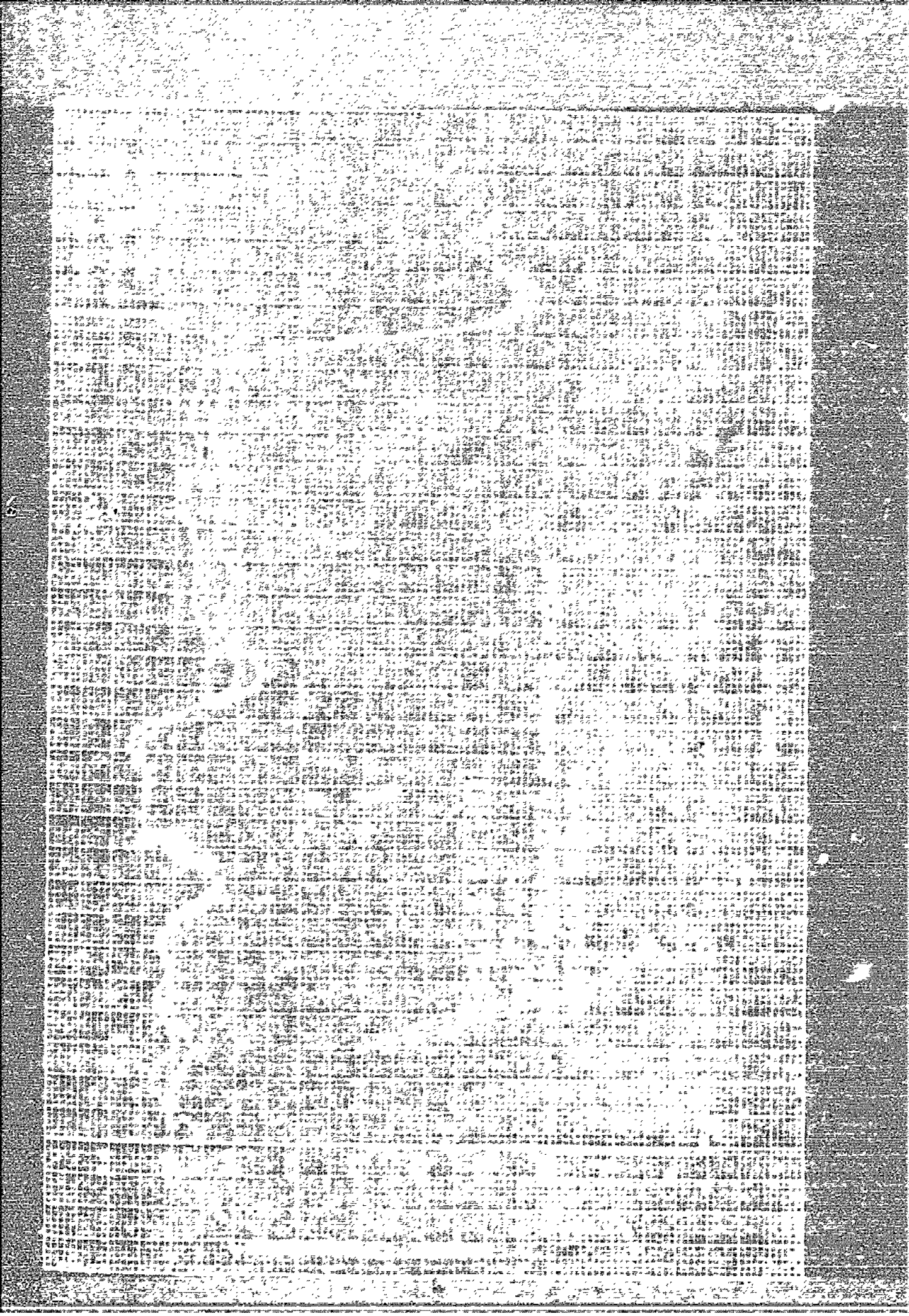


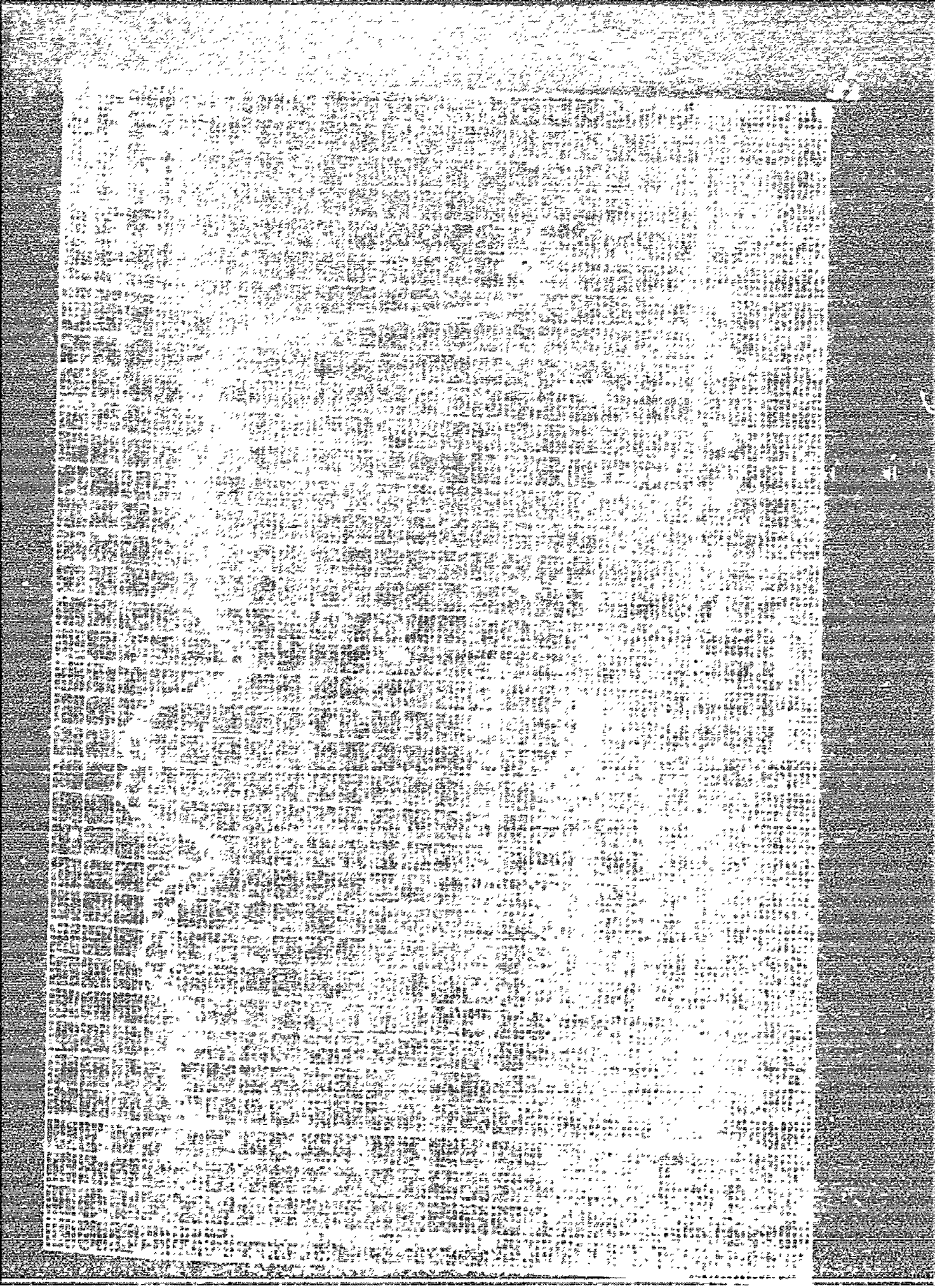












DISCUSSION

Average curves showing the infra-red absorption of pure ergosterol and of ergosterol irradiated with wave lengths longer than 2960 A for different periods of time are shown.

For comparison, the spectra of ergosterol irradiated with unfiltered ultra-violet light and of a-iso-ergosterol and ergostadienone are included. The infra-red absorption bands of ergosterol irradiated with unfiltered ultra-violet light vary so greatly that fairly typical individual curves are used, rather than an average curve.

The iso-ergosterol was prepared by treating an alcohol solution of ergosterol with sulphuric acid according to the method of Heilbron.[50] The ergostadienone was made by heating ergosterol with nickel[51].

INFRA-RED ABSORPTION BANDS OF ERGOSTEROL

Sample	I	II	III	IV	V	VI	VII	VIII	Average Curve
	1.02	1.05	1.05	1.0	1.0	1.0	1.05	1.0	1.0
	1.17	1.15	1.15		1.1		1.15	1.1	
		1.25		1.25	1.2	1.2	1.23		1.25
	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
	1.6			1.55	1.5*	1.5*	1.5*	1.55	1.5*
	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
	2.0			2.0	2.0	2.0	2.0	2.0	2.0
	2.1	2.1	2.1	2.15	2.1		2.1	2.1	2.1
	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3

* - faint band
_ - intense band

INFRA-RED ABSORPTION BANDS OF IRRADIATED ERGOSTEROL = 2960 A

(Approximately 50% converted)

Sample	I	II	III	IV	V	VI	VII	VIII	Average curve
	1.05	1.05	1.05	1.05	1.05	1.0	1.05	1.05	1.0
	1.15	1.15	1.1	1.1	1.1	1.1	1.15		
	1.25	1.25	1.2	1.25		1.25		1.25	1.25
	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
	1.55	1.55	1.5	1.5*		1.55	1.55	1.5	1.5
	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
		1.85							
	1.9	1.9*		1.9	1.9	1.9*	1.9	1.9*	1.9*
	2.05	2.05	2.05		2.05	2.05		2.05	2.05
						2.1	2.15	2.15	2.15*
	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3

INFRA-RED ABSORPTION BANDS OF ERGOSTEROL AND ERGOSTEROL DERIVATIVES

Ergosterol	Irradiated, 50% changed $\lambda = 2960$	Irradiated, 47% changed unfiltered ultra-violet	Iso-Ergosterol	Ergostadienone
1.0	1.0	1.0	1.0	1.0
				1.15
1.25	1.25	1.25	1.25*	1.25
1.4	1.4	1.4	1.4	1.4
1.5*	1.5	1.5*	1.5	
1.7	1.7	1.7	1.7	1.65
1.9	1.9*	1.9	1.9	1.9
2.0	2.05		2.05	
2.1	2.15	2.1	2.15	2.15
2.3	2.3	2.3	2.3	2.3

The Correlation of Absorption Bands and Linkages

The recurrence of certain features among the spectra of chemically related compounds was noted by Abney and Festing in 1881[52] but the work of correlating bands and linkages, most of which has been done on liquids, is still far from complete. Robertson[53] summarizes the evidence upon which correlations are based:

"Repetition of bands or harmonic series of bands is found among the members of homologous series, as for example, the persistent occurrence of certain bands up to 14 μ in the aliphatic hydrocarbons and up to 10 μ among the primary alcohols.

Shift of a band has been observed on changing from a primary to a secondary, and thence to a tertiary alcohol, with the concomitant change of CH_2OH - CHOH - COH.

Identification of a linkage, for example, C-H, with a band system has been inferred from the spectra of two such bodies as chloroform and benzene, which display between 1 u and 3 u a number of bands in common.

Disappearance of a band concurrently with the elimination of a group is among the most cogent lines

of argument in this work. Taking the N-H linkage as an example, Salant found that when the hydrogen atoms of ammonia were all substituted to give the (liquid) tertiary amine, a band at 3 u which had till then persisted in the mono- and diamines, suddenly disappeared. This band also occurs strongly in ammonia and is considered to be the first harmonic of the band of ammonia (gas) at 6.126 μ. It is thus concluded that the degree of freedom which produces this band in ammonia and the substituted ammonias so long as they contain one atom of hydrogen is peculiarly associated with the linkage N-H.

In addition to the observations of coincidences and of the effect of suppression of bands in conjunction with changes in the chemical structure of the substance, attempts have been made to support the identification of a linkage with a band by comparing the calculated with the known value for some other constant, such as the heat of formation."

Ellis[54] states that "An examination of the spectrum curves ----- reveals two facts which suggest that the absorption bands may be arranged in series originating in the longer wave region whose members suffer gradual diminution of intensity and increasingly shorter wave length separations as the series progresses toward the limits of the visible spectrum. These facts are:

1. For investigations in the region between 15 μ and 3 μ the best results are obtained when absorbing thicknesses of 0.1 mm or less are used; for the regions between 3 μ and 2 μ and 2 μ and 1 μ, cells of 1 mm and 10 mm respectively yield the best results, while to bring our clearly the bands between 1 μ and the visible red, cells of about 10 mm thickness are required.

2. There are relatively fewer bands in the longer than in the shorter wave length region.

A single band is rarely significant; series of bands are definitely identified with chemical linkages. The bands below 3 u are overtones of fundamentals which, in most cases occur at longer wave lengths. For this reason, although the bands are crowded and of low intensity this region is significant because a number of the members of a given series are present.

Infra-red absorption bands do not form exactly harmonic series, that is, their frequencies are not exact multiples. Kratzer developed the idea of anbarmonic oscillations in molecules of gases and related the band series with the expression, $\nu = n\nu(1 - nx)$, in which n = 1, 2, --- 10, and x is a small correction factor. Ellis[55] obtained consistent results by applying the Kratzer correction to the band series of organic

molecules.

Gapon related the frequency of a given band, ν_n, to the fundamental frequency, ν_o, by the equation $\nu_n = \nu_o n$, in which n is an integer.[56] Sappenfield used the Gapon correction in his studies of the band series of alcohols. Other correction formulae have been proposed but the Kratzer and the Gapon equations are applicable to the greater number of series.

Rawlins and Taylor[57] find difficulty in accepting long series of related bands: "First, it is not likely that the eighth (or even higher harmonics) would have an intensity capable of measurement; and secondly, in liquids, the bands are usually so broad that the corrections may be merely the amount of experimental error, either in observing such feeble intensities or directly due to differences in calibration."

The C - H Linkage

Puccianti[58] explored a number of benzene derivatives to 2.7 μ and came to the conclusion that all molecules which contain hydrogen directly linked to carbon show a strong absorption maximum at 1.71 μ.[59] Coblentz locates this band at 1.68-1.69 μ, agreeing with Donath. According to Coblentz, hydrocarbons have a character-

istic absorption spectrum with bands near 0.83-0.86 μ, 1.67-1.72 μ, 3.25-3.43 μ, 6.75-6.86 μ and 13.6-14.0 μ. The first striking absorption band occurs near 3.2 μ.

Ellis[55] ascribes a series of bands of decreasing intensity near 2.3 μ, 1.7 μ, 1.38 μ, 1.17 μ, 1.03 μ and 0.9 μ to the characteristic oscillation of the C-H linkage. He believes that these are the third to the seventh harmonics of a frequency found near 6.9 μ by Coblentz. Weak bands near 0.83 μ and 0.76 μ in the spectra of many compounds may represent the eighth and ninth harmonics. From other evidence Salant[61] suggests that the fundamental is located at 3.43 μ rather than at 6.9 μ. Bonino[62] identifies a strong band at 3.43 μ with the C-H linkage.

Smith and Boord[63] studied the spectra to 2.7 μ of a series of ethers, esters and related compounds. They found very little shift due to differences in molecular structure, and mapped a number of bands which form an approximately harmonic series attributed to the C-H linkage. These bands are in very nearly the same positions as those identified by Ellis.

It is probable that some of these bands are double. With good resolution two bands have been noted at 1.7 μ in the spectra of several compounds, while the

breadth of this band in others would indicate a combination of two or more bands.[64]

The Alcohol Groups

The alcohol groups have been investigated by Weniger[65], Puccianti and Coblentz, and more recently by Lecomte and Sappenfield. These investigators do not always agree as to the positions of bands assigned to the OH linkage.

Lecomte[66] identifies two strong bands, definitely separated, at 3 μ and 3.4-3.5 μ. Bands which are probably due to the absorption of the OH linkage occur at 4.3-4.6 μ, 4.8-4.9 μ, 5.2-5.3 μ, 5.98-6.12 μ and 6.98-7.2 μ. Characteristic differences in the spectra of primary, secondary and tertiary alcohols occur beyond 6.9 u.

Coblentz[67] found a shallow band at 2.85 μ which is characteristic of alcohols; he also identifies with the OH group the shallow band at 2.08 μ to 2.14 μ in Puccianti's curves.

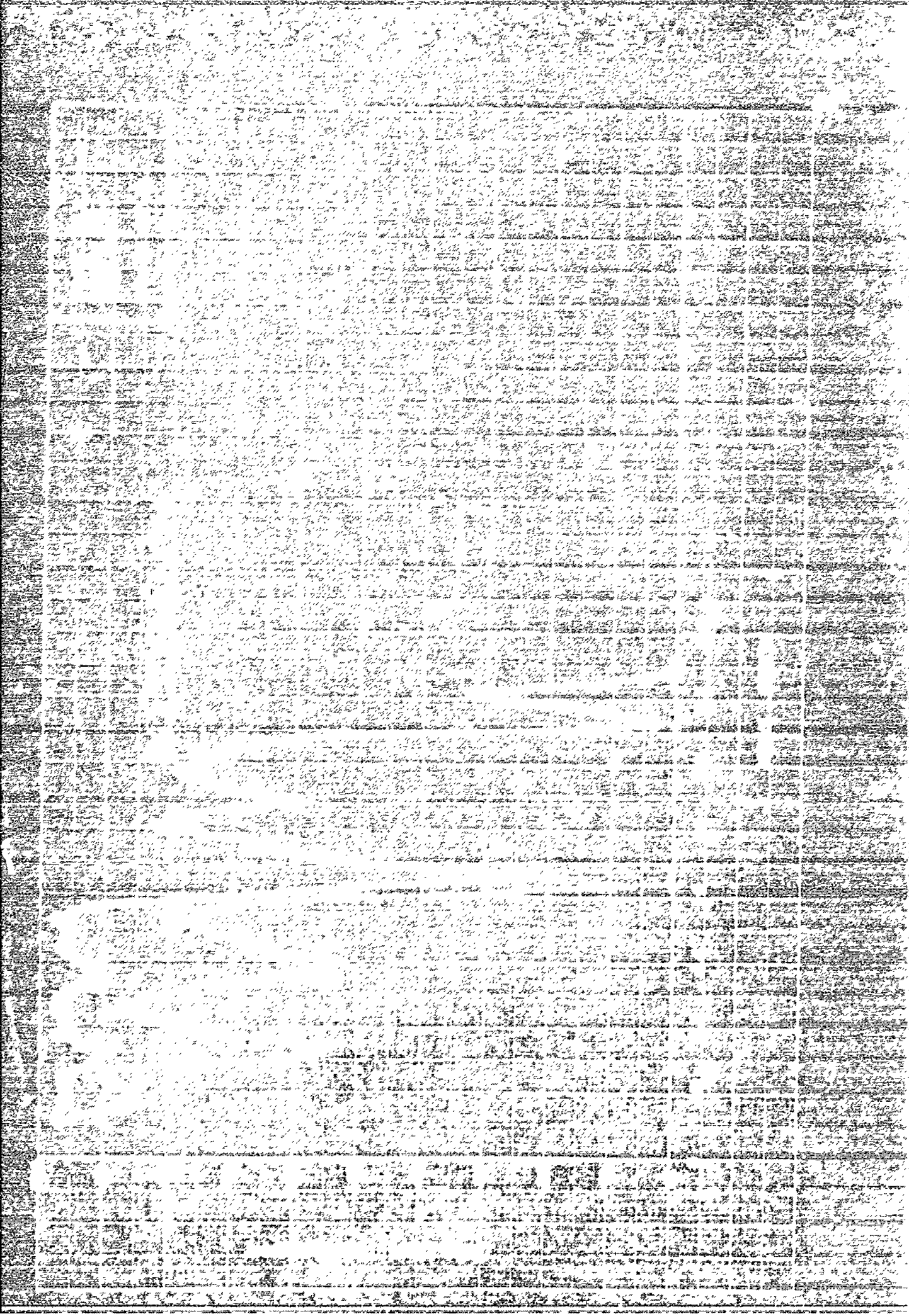
According to Sappenfield[56], the absorption bands due to the OH group occur at 6 μ, 3 μ, 1.55 μ and 1.025 μ. Since bands at 6 μ and 3 μ are found in the spectrum of water they may be assigned to the OH group with some

assurance. The strong band at 1.55 u in the spectra of primary, secondary and tertiary alcohols is undoubtedly due to the OH group. Sappenfield also relates weak bands near 1.02 μ and 1.69 μ to this group although these bands do not appear in the spectrum of every alcohol studied. Many alcohols show a band near 1.69 u.

The Carbonyl Group

From the results of a study of 25 aldehydes and ketones, Lecomte[68] concludes that a band between 5.9 μ and 6.03 u characterizes the carbonyl group. The first overtones of this band occur at 2.9 μ and 3.5 μ. Ellis[69] believes that Lecomte's values are consistently too high and that the fundamental band appears at 5.8 μ.

A summary[69] of the results of Ellis, Lecomte, Henri, Coblentz, Smith and Boord, and Bennet and Daniels shows that a band at 1.9-1.97μ is present in the spectrum of every compound examined; practically all show bands at 5.8-6.0μ and 2.12-2.62 μ; many at 2.9 u and 1.25 u. Ellis believes that a study of the work of these investigators as well as his own results establish at 5.8 u the fundamental band due to the absorption of the $-C-O$ linkage, with overtones at 2.9 μ, 1.9 μ, 1.45 μ, 1.16 μ and 0.97 μ.



Acetophenone shows intense infra-red absorption near 1.9 μ and 1.4 μ which is typical of ketones.

The Benzene Nucleus

Many bands have been attributed to the benzene nucleus, and there is little agreement in the work of different investigators. Dreich[70] lists bands near 2.72 μ, 2.28 μ, 2.18 μ, 1.68 μ, 1.45 μ and 1.179 μ; Barnes and Fulweiler[71], near 1.14 μ, 0.874 μ, 0.71 μ and 0.6066 μ. It is probable that some of these bands are due not to the benzene group itself but to the C-H linkage.

All of the benzene derivatives examined by Puccianti[72] showed absorption maxima at 2.18 μ and 2.49-2.47 μ. Lecomte[73] summarizes investigations of Coblentz, Ellis, Dreich and Marton, which show that these two bands are prominent in the spectra of all simple benzene derivatives. In complex compounds they may be shifted. Other bands characteristic of the benzene group are found at longer wave lengths. A band at 3.25 μ which is characteristic of benzene does not appear in the spectra of cyclo-hexane and cyclo-hexene derivatives. In general cyclo-hexane shows the band spectrum characteristic of the C-H linkage.

Conjugated ring compounds have not been extensively investigated. Lecomte records a band at 5.67 μ for napthalene derivatives.[74] Bell states that the spectrum of napthalene resembles that of benzene.

SUMMARY OF ABSORPTION BANDS

LINKAGE						
C H	.9	1.02	1.17	1.4	1.7	**2.3** (E)
O H		1.025 (S)		1.55 (S)	2.08-2.14 (P)	2.95 (C)
					1.69	1.94 (S)
= C = O	.97		1.16	**1.45**	1.9	2.12-2.62
Benzene						2.18, 2.48 (P)

- intense band

* faint band, not present in all compounds examined

E - Ellis

C - Coblentz

L - Lecomte

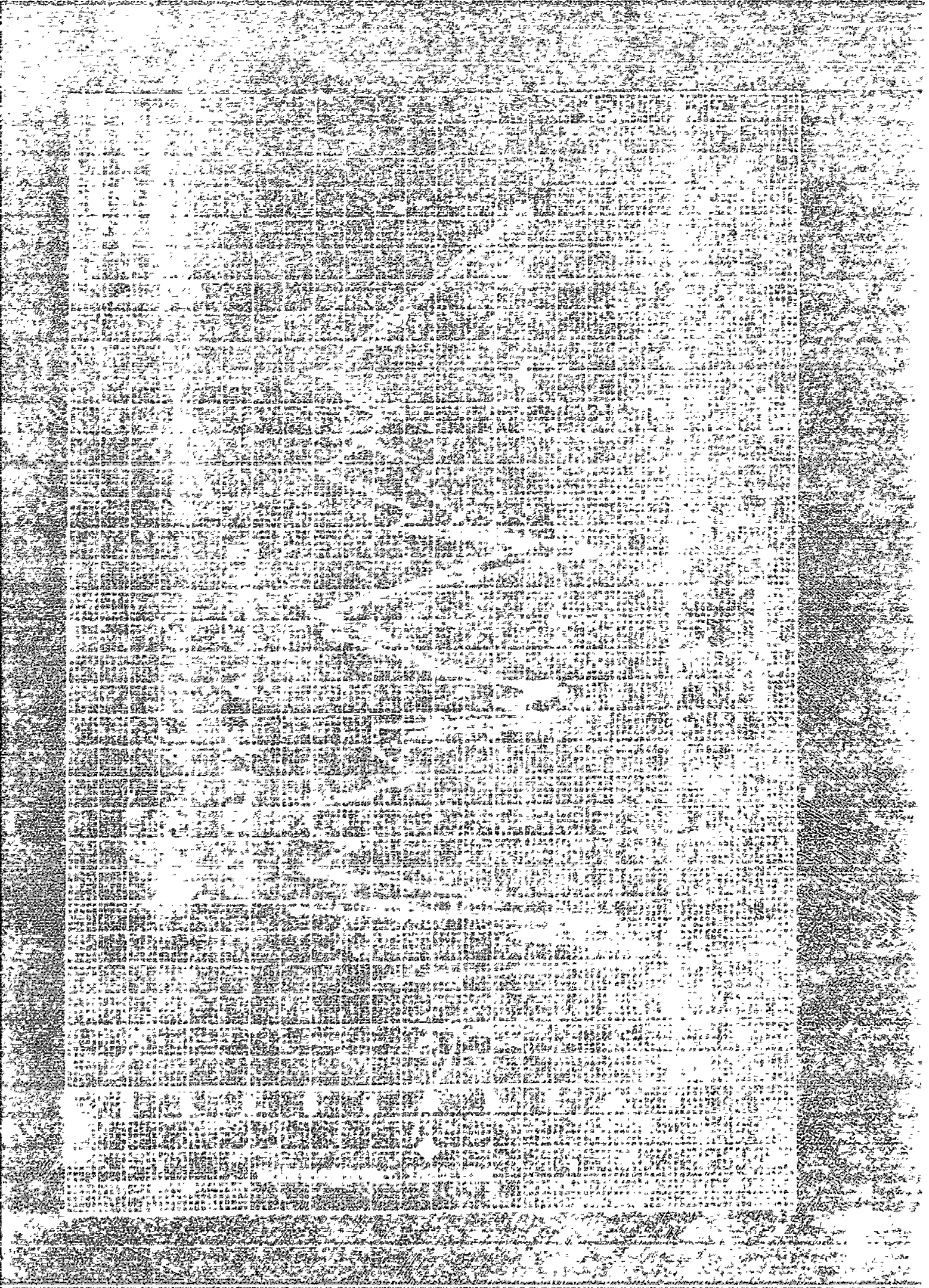
P - Puccianti

S - Sappenfield

Isomers

A study of isomeric compounds shows the influence of the arrangement of atoms in the molecule. Isomers containing different groups, for instance an ether isomeric with an alcohol, have distinct spectra, while stereoisomeric compounds such as d and l pinene have identical spectra. Coblentz[75] states that the spacial arrangement of atoms in the molecule has no effect upon the infra-red spectrum.

Lecomte[76] reviews studies of isomers, particularly of esters isomeric with organic acid. Such isomers show different absorption curves, while butyl and iso-butyl alcohol have very similar curves. Lecomte finds that the chief absorption bands of isomers occur at identical positions while less intense bands characteristic of the individual compound occur in different positions.



The Absorption Spectra of Ergosterol and Irradiated Ergosterol

The absorption bands near 1.0 μ, 1.15 μ, 1.4 μ, 1.7 μ and 2.3 μ are quite evidently members of the series of bands associated with the C-H linkage.

Those at 1.55 μ and 2.05 μ are identified with the alcohol group. The band at 1.99 μ and the faint band at 1.25 μ which appear both in the spectra of ergosterol and irradiated ergosterol are probably associated with the alcohol group since bands in these positions are found in the spectrum of almost every alcohol studied by Sappenfield. It must be noted that these bands have also been assigned to the ketone group by Ellis.

The band near 2.15 μ is possibly identified with the arrangement of carbon atoms in the nucleus. This band appears in the spectra of all of the ergosterol and cholesterol derivatives thus far examined.

Any indications of diminution of the alcohol bands or in the development of bands which might be associated with the ketone group were carefully watched. The development of new bands is not indicated in the series of preparations irradiated for periods of 1, 2, 3, 4, 5 hours. Comparisons will be based on the spectra of ergosterol irradiated for five hours in which about 50% of the

ergosterol was changed.

The bands at 1.55 μ and 2.05 μ are still present in the irradiated ergosterol, indicating the persistence of the OH group. Sample I, irradiated for six hours, with about 60% of the ergosterol changed, shows these bands in undiminished intensity. The fundamental ketone band occurs at 5.8 μ with overtones at 1.9 μ and 1.4 μ in the region investigated. Unfortunately these overlap bands assigned to the C-H linkage and definite conclusions must await an investigation of spectra beyond 5.8 u. However, the absorption of irradiated ergosterol is not increased at 1.9 μ and 1.4 μ as would be expected if a ketone group were developing as a result of irradiation.

In almost every case curves of ergosterol irradiated with unfiltered ultra-violet light show a fairly intense peak at 1.9 μ. Chemical and biological investigations and studies of the ultra-violet spectra have shown that the products of irradiation with long ultra-violet wave lengths are different from the products of short wave irradiation. The differences in the infra-red spectra suggest that a ketone derivative may be the result of irradiation with ultra-violet light of short wave length. It is quite possible that a large part of the evidence for the presence of a ketone in irradiated ergosterol is based upon studies of short wave irradiated

preparations.

Ergostadienone, a ketone derivative of ergosterol, shows intense peaks at 1.9 μ and 1.4 μ and a shift of the 1.7 μ band. The general form of the curve of ergosterol irradiated with ultra-violet light of wave lengths longer than 2960 A closely resembles that of pure ergosterol and iso-ergosterol; it does not resemble the curve of ergostadienone.

CONCLUSIONS

It is planned to extend this investigation farther into the infra-red region in order to study the more intense and widely separated fundamental bands of a number of ergosterol and cholesterol derivatives. Definite conclusions must not be drawn before more work has been done.

This preliminary investigation does indicate that the alcohol group persists in ergosterol irradiated with wave lengths longer than 2960 A while bands usually associated with the ketone group do not appear. The resemblance of the curve of irradiated ergosterol to that of pure ergosterol and of isoergosterol suggests that the product of irradiation is an isomeric form of ergosterol.

SUMMARY

A preliminary investigation of the infra-red spectra between .8 u and 3 u of ergosterol and irradiated ergosterol indicates that the alcohol group does not diminish and that the ketone group does not develop as a result of irradiation with ultra-violet light of wave lengths longer than 2960 A.

The general form of the absorption curve of irradiated ergosterol resembles that of ergosterol and iso-ergosterol.

These findings suggest that vitamin D is an isomer of ergosterol.

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