CLXV. THE ISOLATION OF SPERMINE PHOSPHATE FROM SEMEN AND TESTIS.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London, and the National Institute for Medical Research, Mt. Vernon, Hampstead, N.W. 3.

(Received October 3rd, 1924.)

In the course of experimental work on "sperma crystals," extending over the last twenty years, I made an exhaustive study of the literature, as an outcome of which a new chapter must be added to the already remarkable history of an equally remarkable substance. According to the statements of the text-books, accepted by all writers on the subject, these peculiar crystals were discovered by Böttcher [1865] in human semen and they have since been known as "Böttcher's crystals." Long before Böttcher, however, they were accurately described by Vauquelin [1791], and even this distinguished observer only rediscovered the crystals which, more than a hundred years before, had been seen and depicted by Leeuwenhoek [1678]. The passage describing the crystals occurs at the end of the famous communication to the Royal Society in which Leeuwenhoek announces the discovery of spermatozoa. The rough marginal sketch (see Fig. 1), like many others drawn by Leeuwenhoek in his letters to the Royal Society, has suffered somewhat in reproduction by the hand of the engraver, but in the following few words, he gives an admirable description of the "glittering, translucent" crystals, including even their correct measurement¹: "Et cum praedicta materia paucillum temporis steterat, in ea observabantur trilaterales figurae ab utraque parte in aculeum desinentes, quibusdam longitudo minutissimae arenae, aliquae aliquantulum majores ut fig. A. Praeterea, adeo nitidae ac pellucidae, ac si crystallinae fuissent."

Fig. 1

Vauquelin [1791], evidently unaware of Leeuwenhoek's previous observation, characterised the crystals as possessing "la forme d'une solide à quatre pans, terminés par des pyramides très allongées, à quatre faces." He was the first to attempt their chemical investigation, and the analysis of an obviously impure preparation led him to the diffidently expressed view that they repre-

¹ Leeuwenhoek's "smallest grain of sand" measures according to his own scale approximately one-hundredth of an inch, i.e. 0.25 mm. For this and other information regarding Leeuwenhoek's work, I am much indebted to Mr Clifford Dobell, who also kindly lent a copy in his possession for the reproduction of the above-quoted passage.

sented a hitherto unknown crystallised form of calcium phosphate. Subsequently Berzelius [1833] mentions the crystals in his investigation of semen and, without attempting their analysis, suggests that they consist of ordinary ammonium magnesium phosphate crystals, as so frequently found in other animal fluids¹. Probably on Berzelius' authority the text-book writers of the period accepted this view, and the crystals are thus fully described by C. G. Lehmann [1853]. Their existence might therefore have been known to Böttcher [1865], when he "rediscovered" and described the crystals under the title "Farblose Krystalle eines eiweissartigen Körpers aus dem menschlichen Sperma." Böttcher's unconvincing evidence for the protein nature of the crystals, based on microchemical reactions, was accepted by subsequent text-book writers, as for instance by Kühne, who assumes them to be vitellin, and by Hoppe-Seyler who compares them to aleurone grains of seeds.

Ph. Schreiner [1878] was the first to recognise the true nature of the crystals. In a classical paper, which unfortunately lacks essential experimental details, Schreiner came to the conclusion that the crystals were the phosphate of a new organic base. The material obtained by him from semen was evidently just enough for an analysis and a study of the properties of the base. With this knowledge he succeeded in demonstrating that a base, yielding the same characteristic insoluble phosphate, is widely distributed in the animal organism. From the analysis of the hydrochloride he deduced the formula C_2H_5N , a formula with which his analytical figures for the phosphate are not in agreement, as pointed out by Ladenburg and Abel [1888]. It will be shown later [see Dudley, M. C. Rosenheim and O. Rosenheim, 1924] that Schreiner's analysis of the hydrochloride is faulty, whilst his figures for the phosphate correspond to the correct empirical composition of the base.

Schreiner did not name the new substance, and it was known as "Schreiner's base" or "spermatin" for some time [see Thudichum, 1879; Fürbringer, 1881]. The name "spermatine" had been previously used by Berzelius [1833] for a specific protein of semen, and the name "spermin," apparently first applied to the base by Ladenburg and Abel [1888] has since been in use.

It is a remarkable fact that no investigator since Schreiner has again been able to isolate spermine phosphate from semen, so that quite recently Wrede and Banik [1923], after an unsuccessful attempt, consider it doubtful whether the phosphate will ever be obtained again. Nor have the attempts to verify Schreiner's statements of the occurrence of spermine in testis been successful [Majert and Schmidt, 1890; Merck's Chem. Works, Parke Davis & Co., see Jürgens, 1890]. Its existence in animal organs has even been doubted by Wrede and Banik, who were evidently unaware of the recent confirmatory work of M. C. Rosenheim [1917]².

¹ This view is still held by at least one modern worker, who also denies the existence of spermine in semen [Slowtsoff, 1902, 1916].

² Since it has now been established by M. C. Rosenheim [1917] that spermine passes into the lysine fraction, it is interesting to find that various authors came very near to its rediscovery in spleen and pancreas. Gulewitsch and Jochelson [1900], examining the lysine fraction of an

The only author who claims to have isolated spermine phosphate from testis and other organs is the Russian physiologist, A. von Poehl [1891]. His chemical investigation, incomplete and vague as to details, stands discredited together with his pharmacological and clinical work, on account of his persistent commercial exploitation of the assumed valuable therapeutic properties of spermine [see Poehl's "Spermin Theorie," 1898, 1906]. Although the commercial material, "Sperminum Poehl," of which I examined a sample recently, contained only traces of spermine, there is no doubt that Poehl had actually isolated spermine phosphate and that his single analysis of the chloroplatinate, to which he ascribed the formula $C_5H_{14}N_2 \cdot H_2PtCl_6$, is correct. Through the kindness of Prof. Halliburton, I came many years ago into the possession of a specimen of spermine phosphate which had been prepared and presented to him by the late Prof. Poehl. Crystallographically and chemically Poehl's specimen is identical with the product prepared by me from semen and testis.

In my own earlier attempts to prepare spermine phosphate from semen, I invariably failed, as no doubt other observers before, and Wrede since has done, to isolate the crystals. This failure was not due to the absence of the substance, but is inherent in the method as described by Schreiner, as will be shown in the experimental part. I succeeded finally by adopting a slight, but essential, modification, suggested by the experience with Vauquelin's original sedimentation process. The crystals can also be obtained directly from semen by dialysis. During the progress of the work on the isolation of spermine from animal organs which is described in the subsequent paper [Dudley, Rosenheim and Rosenheim, 1924], the observation was made that the free base is easily extractable from alkaline solution by butyl alcohol, and further that it can be distilled with steam. Methods of isolation from semen based on these observations will be described.

It will further be shown that the presence in testis of spermine phosphate as such can easily be demonstrated. The identity of the various preparations from semen and testis with those from pancreas, spleen, yeast, etc., has been proved by their crystallographic analysis, by their melting points, and by the preparation from them of the picrate, picrolonate, chloroplatinate and chloroaurate of spermine.

EXPERIMENTAL.

Leeuwenhoek's discovery may be easily verified and the striking phenomenon of the appearance of the characteristic crystals can best be followed under the microscope by mounting a drop of fresh semen on a slide under a

extract of ox spleen, obtained 20 mg. of a base which gave a microcrystalline precipitate with phosphoric acid, the identity of which with spermine they were unable to decide owing to lack of material. Kutscher and Lohmann [1904, 1905] state that they repeatedly obtained from the lysine fraction of autolysed pancreas the picrolonate of an unidentified base, which according to their description and nitrogen determination may now probably be identified with spermine picrolonate. Werigo's [1892] "cadaverine picrate" from pancreas was very likely spermine picrate.

coverslip. The shortest time of their appearance is, according to my observations, from forty to sixty minutes, after the semen has undergone the peculiar rapid change from the viscid tenacious condition into a thin watery fluid. Since spermine phosphate as such exists in testis and other organs, its crystallisation from semen is most easily explained by changes in temperature and hydrogen ion concentration.

With regard to their size, my observations agree with those of previous observers. Their average length is 0.25 mm., but crystals up to 2 mm. in length may occasionally be seen.

(1) *Isolation from semen.*

It may safely be said that it was not the difficulty of procuring material alone, but rather the uncritical adherence to Schreiner's directions which caused the failure of subsequent observers to isolate the crystals, and thus to doubt their existence [see Wrede and Banik, 1923]. It is unfortunate that Schreiner, in ignorance of the simple procedure adopted by Vauquelin, chose a method which must necessarily lead to failure when carried out exactly as described by him. As the reasons for this statement became apparent only after the successful isolation of the crystals by other methods, it will be advisable to follow the historical sequence.

(a) *Modification of Vauquelin's method.* Vauquelin observed that the crystals, being heavier than the fluid in which they are suspended, fall to the bottom when fresh semen is allowed to stand for some time in a narrow vessel. He decanted the supernatant fluid and washed the crystals by decantation with cold water, in which they are insoluble. By this simple process he succeeded unknowingly in removing the peculiar protein of semen, which prevents the isolation of the crystals by Schreiner's method. On the other hand, this crude method must necessarily yield a very impure preparation, since the sediment is seen, on microscopical examination, to consist of a mass of crystals entangled with spermatozoa and debris. By adopting, however, the principle of Vauquelin's method, the crystals can be easily isolated in a fairly pure condition.

Fresh semen is allowed to stand in a centrifuge tube for some hours. The contents of the tube are then well mixed with an equal volume of 0.9 % sodium chloride solution, and the sediment centrifuged off. It is freed from soluble proteins by repeatedly washing it in the centrifuge with salt solution, subsequently with water and with alcohol. It is now boiled up with alcohol, centrifuged when cold, and finally washed with ether. A white powder remains which can be easily removed from the tube and extracted with boiling water. A perfectly clear filtrate, free from proteins, results, from which the crystals separate on cooling, if necessary after suitable concentration and the addition of a few drops of alcohol. Yield from 4.5 cc. semen: 6 mg. = 0.14 %. From 25 cc. semen, collected and worked up in portions of about 5 cc., were obtained 32 mg. spermine phosphate = 0.13 %.

(b) *Modification of Schreiner's method.* The method as described by Schreiner is shortly as follows: "Fresh semen is boiled with alcohol in order to coagulate the proteins. After some hours the precipitate is filtered off and dried. The substance is finely ground and extracted with warm water, to which a few drops of ammonia have been added. Only traces of proteins pass into solution, whilst the phosphate is completely extracted and crystallises out in its peculiar forms on concentration of the extract. In a quantitative experiment the dry residue of semen yielded 5.2 % of the crystals."

There is no difficulty in repeating the first part of Schreiner's directions, which are only a confirmation of Vauquelin's and Berzelius' previous observations. Semen is precipitated by alcohol as a perfectly white stringy mass, which, when filtered and dried, amounts to about 10 % of the semen. When this precipitate, however, is ground up with water it swells up and forms a colloidal solution. Contrary to Schreiner's statement, it is found that on warming or boiling the gelatinous fluid practically the whole of the protein goes into solution. Filtration through paper, with or without pressure, soon stops, and as a rule only a few cubic centimetres of filtrate are obtained. On slow evaporation of the filtrate transparent membranes form on the surface of the fluid, drying to varnish-like films in which well-formed crystals of spermine phosphate are embedded.

The following slight, but essential, modification of Schreiner's method led to the isolation of the crystals in a fairly pure condition. The dried alcohol precipitate is finely ground and treated with water at room temperature for a few hours until a homogeneous suspension results. On centrifuging, a colourless protein solution and a white sediment are obtained. The solution is decanted and the treatment with cold water repeated until the supernatant fluid no longer yields a precipitate when poured into five volumes of alcohol. Success depends on the efficient removal of the water-soluble protein, and at least five extractions are necessary. The sediment is then washed in the centrifuge with cold alcohol, afterwards with boiling alcohol, and finally with ether. There remains a white powder amounting to 30-40 % of the weight of the original substance. On extraction with boiling water, this powder gives a water-clear filtrate, free from protein, from which the characteristically curved spindle-shaped crystals of spermine phosphate deposit on cooling. The yield is approximately the same as that obtained by the previous method. From 14.5 cc. semen, 25 mg. of the phosphate = 0.17 % were obtained.

It is evident from these experiments that human semen contains a peculiar protein soluble in cold water and not coagulated on boiling. By pouring the watery extracts, obtained at room temperature as above, into five volumes of absolute alcohol a voluminous white precipitate was obtained, which was centrifuged off, and washed with alcohol and ether. On drying it shrank to a horny mass which could be easily powdered. The fine white powder is easily soluble in water, reacting strongly alkaline to litmus. The solution is not coagulated by boiling, even when acidified with acetic acid. It gives the

biuret reaction and is precipitated by picric acid as well as by other alkaloidal reagents, in acid and alkaline solution. In several quantitative experiments its amount varied from 35–40 % of the original dry semen. According to its reactions, this protein belongs to the group of protamines which have been isolated by Kossel and his co-workers from the spermatozoa of fishes. The existence of this substance as a specific constituent of human semen had already been noticed by Berzelius [1833], who called it "spermatine." More recently Posner, and especially Slowtsoff [1902, 1916] took the substance for a "propeptone" or albumose. Hofmann [1909] isolated by Kossel's method a small amount (0.36 g.) of a substance from human semen, which on the basis of somewhat scanty evidence, he considers to be protamine sulphate.

Schreiner was clearly unaware of the existence of this protein, and it is difficult to understand from his description how he avoided its interference with the isolation of the crystals. Perhaps the surprisingly large yield of spermine phosphate (5 % of the dry semen), which he claimed to have obtained, was due to the admixture of protamine.

(c) *Dialysis.* Although pure spermine phosphate is only slightly soluble in water, it is possible to separate it from semen by dialysis. For this purpose it is advisable to use "dry semen" prepared as above by precipitation with alcohol, since choline and other dialysable substances are thus removed. The powder is boiled with about ten volumes of water, in order to ensure sterilisation and to bring the phosphate into solution. A membrane condom (sheep's caecum) was used as a dialyser, and toluene as an antiseptic. Dialysis is very slow, and had to be continued for eight days, the dialysate being removed every 24 hours. After concentration, a small amorphous precipitate was removed by filtration, and crystallisation started by the addition of a few drops of alcohol. Particularly well-formed crystals of spermine phosphate were obtained. The alcohol-precipitate from 90 cc. semen yielded 0.20 g. = 0.22 %.

(d) *Butyl alcohol method.* 50 cc. semen were precipitated with alcohol as described above. The dry powder was boiled up with 70 cc. water, faintly acidified with acetic acid, and precipitated with 7 cc. of a saturated lead acetate solution. The precipitate settled on slight warming, and was centrifuged off. The opalescent solution, which gave no further precipitate with basic lead acetate, was freed from lead with sulphuric acid and filtered through kieselguhr. The clear filtrate was made strongly alkaline and extracted with butyl alcohol in an apparatus for continuous extraction, made entirely of glass. Four extractions, lasting six to eight hours each, were made. The colourless extracts were precipitated with a 25 % solution of phosphotungstic acid in butyl alcohol. The white precipitate was allowed to settle and washed four times by decantation with acetone. After filtration and drying *in vacuo*, the phosphotungstate was obtained as a white voluminous powder, weighing 3.6 g. This was decomposed by baryta in the cold, filtered and freed from excess of baryta by CO_2 . The final filtrate measured 30 cc. One-third of it was acidified

with dilute phosphoric acid to Congo red, and the remaining two-thirds added. The resulting solution reacts amphoteric to sensitive litmus paper, and deposits well-formed crystals of spermine phosphate on standing. The crystallisation was hastened and completed by the addition of 10 cc. alcohol. Yield 0.12 g. = 0.24 %.

(e) *Steam distillation.* The alcohol precipitate from 5 cc. of semen was prepared as previously described, dissolved in 10 cc. of strong alkali and steam distilled in a Claisen flask. Distillation was continued until a drop of the distillate no longer reacted with potassium bismuth iodide or picric acid. (The picrate from another experiment had the correct melting point of spermine picrate.) Altogether 300 cc. of the distillate were collected and evaporated nearly to dryness. The residue was dissolved in 1 cc. water, and 0.5 cc. of a 20 % solution of ammonium phosphate was added. Crystallisation began immediately on scratching with a glass rod, the phosphate depositing as a heavy white powder. The phosphate may also be prepared by neutralisation of the distillate with phosphoric acid, as described under (d). Yield 14 mg. = 0.28 %.

As regards the choice of methods, the steam distillation is the most rapid and satisfactory as to quality and quantity of product. The purely physical methods described under (a), (b), and (c) are tedious and yield on the whole a somewhat less pure product. The crystals obtained by these methods usually show besides the characteristic spindle-shaped curved crystals, a certain proportion of prismatic crystals, as frequently seen in sperma itself. This tendency persists on repeated recrystallisation. Fig. 2 (Plate VIII) shows typical crystals of spermine phosphate prepared from semen, testis, pancreas and yeast.

(2) *Spermine phosphate in testis.*

The existence of spermine in testis has been definitely denied by Majert and Schmidt [1890] in connection with their work on piperazine and its supposed identity with spermine¹. They worked up fifty bull's testes by Schreiner's method without obtaining a trace of the base. Their failure is easily explained by their unfamiliarity with the peculiar properties of spermine phosphate and the difficulty of obtaining it crystallised except under certain well-defined conditions.

The presence of spermine phosphate in testis can be easily demonstrated when the tissue is "plasmolysed" by means of ether vapour. A highly concentrated "exudate" of the water-soluble cell contents is thus obtained, from which spermine phosphate and proteins are precipitated by alcohol. Their separation is carried out as previously described. The method is too tedious for the isolation on a large scale, especially as the yield is only 10–15 mg. from six testes (2 kg.).

¹ See Poehl [1898] for details of this unfortunate episode in the spermine history which caused much confusion, but is now only of secondary interest.

Böttcher had already noticed crystals resembling spermine phosphate on the surface of old pathological-anatomical preparations, and this observation was confirmed by Schreiner¹. I repeated this experiment by suspending a bull's testicle, weighing 340 g., in 700 cc. 96 % alcohol in a stoppered bottle. Three weeks later crystals of spermine phosphate, singly and arranged in rosettes, could be seen under the microscope in the scummy deposit from the surface of the organ. After three months macroscopic crystals of 3–4 mm. in length had formed. The crystals were scraped off from the tissue and the sides of the bottle, boiled up with alcohol and the residue extracted with water. Typical crystals of spermine phosphate, weighing 6 mg. = 0.002 % were obtained. The yield is only one-third of that obtained by the methods to be described later.

It seems justifiable to conclude from these experiments that spermine phosphate exists as such in the organ, unless we assume it to be formed post mortem by enzymic processes in spite of the presence of ether and alcohol.

The identity of the spermine phosphate preparations from semen and various animal organs, etc., has so far not been proved by strictly chemical methods. Schreiner unfortunately failed to give its melting point; he merely mentions its behaviour when heated on a platinum foil and says that the crystals "liefern bei circa 170° C. eine etwas gebräunte, beim Erkalten wieder fest werdende Schmelze." This observation forms the basis of the statement, transmitted in successive generations of text-books, that the melting point of spermine phosphate is 170°. In his monograph, Poehl [1898] gives it at 130–135°, presumably for a testis preparation. In one of his earlier communications [1892], however, occurs the statement that the dry substance, prepared from pancreas, melts between 230–240°.

The melting points of spermine phosphate, prepared from semen, and of two other preparations, from testis and yeast respectively, were taken simultaneously on the same thermometer. The specimens showed identical behaviour, frothing up and melting between 230–235° (uncorr.), finally running down the capillary and solidifying to a white amorphous mass on cooling. A mixture of the phosphates from semen and pancreas showed no depression of the melting point.

On heating in a test-tube all the preparations give a strong pyrrole reaction with a strip of pine wood dipped in hydrochloric acid. They all show the ninhydrin reaction [M. C. Rosenheim, 1917].

One of the most characteristic salts of spermine is the picrate. Like the phosphate, it is extremely insoluble in cold water, and crystals of more than 1 cm. in length were obtained from the phosphates prepared from semen by any of the methods described above. It melts at 249–250° with frothing. A mixture with the picrate from pancreas showed no depression of the melting point. The crystallographical examination of spermine picrate proves inci-

¹ Böttcher's statement that the crystals forming in egg-white on standing are identical with spermine phosphate was not confirmed by M. C. Rosenheim [1917].

dentally that this is the substance responsible for Barberio's semen reaction with picric acid, a reaction of diagnostic value in forensic medicine, which has been widely discussed without receiving a conclusive explanation. (For literature see Littlejohn and Pirie [1908], and especially B. Baecchi [1912].)

I further prepared the picrolonate (m.p. 288–289°), the chloroplatinate (m.p. 245°), and the chloroaurate (m.p. 225°)¹. Their melting points are identical with those of the salts prepared from pancreas and other organs to be described fully in the joint communication with H. W. Dudley and M. C. Rosenheim.

Mr T. V. Barker, of the Mineralogical Department, University of Oxford, has kindly carried out the crystallographic examination of the various salts.

REPORT BY T. V. BARKER.

The colourless crystals of spermine phosphate obtained from semen are identical in all their geometrical and optical characters with those derived from pancreas. The form of the crystals is generally lenticular. In absence of any straight edges the long axis of the crystals was selected as a line of reference, and gave an extinction angle of 22° between crossed Nicols. The refractive index of the ray making the smaller angle with the crystal axis is 1.50, whilst the other ray gave a value 1.53. The specific gravity is 1.42.

The picrates also gave identical constants. The crystals are yellow flattened prisms with straight extinction. The refractive index of the ray vibrating along the prism has a value 1.503. The other ray has a much higher value (possibly in the neighbourhood of 1.9), and could not be determined by immersion methods.

The chloroplatinates were obtained in measurable crystals, and thus allowed a most rigorous proof of identity. The salt of pancreatic origin crystallises in brilliant-red monoclinic prisms of the habit shown in Fig. 3, the forms developed being *m* (110), *r* (101), *q* (011) and *s* ($\bar{2}$ 31). The elements $a : b : c = 0.5672 : 1 : 0.7840$, $\beta = 116^\circ 44'$ were computed from the following mean values derived from two crystals:

Fig. 3

	<i>m</i>	<i>r</i>	<i>q</i>	<i>s</i>
ϕ	*63° 8'	90° 0'	*32° 44'	312° 17' (0)
ρ	90 0	63 57 (2)	*43 0	74 3 (– 1)

In polarised light the extinction on *m* makes an angle of 22° with the prism edge. An optic axis is visible through *m* on the edge of the field.

¹ In a recent paper Wrede [1924] describes the chloroaurate (m.p. 216–218°) of a base obtained from human semen and gives analyses of some salts derived from it. He considers it probable that this base is identical with Schreiner's base. Proof of this identity would only be afforded by the preparation of the characteristic phosphate, a task which apparently Wrede has not yet been able to accomplish. In the light of the work of H. W. Dudley, M. C. Rosenheim and O. Rosenheim [1924], however, it is evident that the base isolated by Wrede from semen is identical with that obtained by these authors as a phosphate from various animal tissues and which has been definitely identified with spermine. This conclusion is confirmed, in spite of various discrepancies in connection with melting points and analysis, by the agreement shown in the molecular weight of derivatives of the two bases.

A minute crystal of the chloroplatinate obtained from semen was also measured. Although its mass was only 0.0004 g., the crystal gave excellent reflections on the two-circle goniometer. The following measurements reveal a degree of approximation with the foregoing, which is but seldom exhibited by crystals of the same substance:

	<i>m</i>	<i>r</i>	<i>q</i>	<i>s</i>
ϕ	63° 7'	90° 0'	32° 45'	312° 14'
ρ	90 0	64 0	42 59	74 2

SUMMARY.

1. Contrary to accepted statements in the literature, the "sperma crystals" were not discovered by Böttcher (1865), but had been previously described by Vauquelin (1791). The latter observer only "rediscovered," apparently independently, a fact originally observed by Leeuwenhoek in 1678.
2. Several new methods for the isolation of these crystals (= spermine phosphate) from semen are described, Schreiner's original method (1878) having been found unworkable.
3. Spermine occurs in testis, in which it is present as spermine phosphate.
4. Chemical and crystallographical evidence is brought which proves the identity of spermine from semen with the base obtained by similar methods from various animal organs and also from yeast.

My thanks are due to the Medical Research Council for permission to complete this work in the National Institute for Medical Research, to Dr Dale, the head of the Department concerned, and to Dr Dudley, who placed the facilities of his laboratory at my disposal. Mr W. W. Starling has given me much valuable assistance.

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(1) From Semen

(2) From Testis

(3) From Pancreas

(4) From Yeast

Fig. 2. Photomicrographs of Spermine Phosphate from various sources.

Magnification $50\times$