The antigen prepared by this method gives positive biuret and Millon's tests and a positive xanthoproteic reaction. It is not coagulated by heat. Intracutaneous test in guinea pigs which have been previously infected with different strains of brucella organisms showed that a definite skin reaction lasting more than 48 hours can be produced when 0.005 mg of the purified protein is injected. Larger doses may produce necrosis or even death of the animals, depending on the degree of sensitization. Sera of rabbits inoculated with *Brucella* give a positive precipitin test and a positive complement fixation test when purified brucella protein is used.

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Alteration of Chromosome Sensitivity to X-rays with NH_4OH.

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Previous experiments have led the writer to attribute the formation of chromosome abnormalities by X-rays to the action of electrons on the positively charged materials of the chromosomes.[1, 2] According to this hypothesis, it is to be expected that if the sign of the electrical charges carried by the X-ray-sensitive materials of the chromosomes could be changed, the sensitivity of chromosomes to alterations by X-rays would be very much reduced. If the pH of the medium surrounding the sensitive substance could be shifted to the alkaline side of its isoelectric point or to the alkaline side of the pK of those of its constituent groups responsible for the X-ray reaction, the ionization of these positively charged groups would be suppressed and only the negatively charged groups would be left ionized. Thus it is to be expected that penetrating bases, if they are strong enough to change the intracellular pH sufficiently, should reduce the sensitivity of the chromosomes to X-rays. Penetrating acids, on the other hand, should have no effect unless the pH was shifted to such an extent that new chromosome materials or their constituents were thus brought to the acid side of their isoelectric points and therefore rendered electropositive. Substances behaving in this manner would most likely be proteins. Furthermore, if the pH of the intracellular medium surrounding the chromosomal ma-

[1] Marshak, A., *Proc. Nat. Acad. Sci.*, 1937, **23**, 362.

[2] Marshak, A., and Hudson, J. C., *Radiology*, 1937, **29**, 669.

terials be taken as approximately 7.0, there are only two types of proteins that would carry an appreciable positive charge at this pH, the protamines and the histones. These were also the proteins indicated as the reactive ones by calculations of the size of the "sensitive volume" of the chromosomes.[1]

To determine whether the sensitive material behaves in the manner predicted by the hypothesis, the following experiments were performed: Seedlings of *Allium cepa* (onion) were brought into atmospheres of known CO_2 concentrations where they were kept for half an hour in order to come to equilibrium, and then irradiated with X-rays in the atmospheres with which they had been equilibriated. The normal and abnormal anaphases were then counted at 3 hours after irradiation. Using 11, 20, 40, 60, and 80 volumes % CO_2, no significant effect on the sensitivity of the chromosomes was observed. The differences in the percent abnormal anaphases at the different concentrations were no greater than might be expected from errors in the determination of the dose of X-rays. Therefore, if there are any other proteins in the chromosomes capable of reacting to X-rays, they must have isoelectric points lower than that represented by the intracellular pH produced by an atmosphere of 80% CO_2.

In sharp contrast to the results obtained with CO_2, it was found that dilute solutions of ammonium hydroxide in distilled water would markedly alter the sensitivity of chromosomes to X-rays. Thirty to 40 seedlings of *A. cepa* were immersed in solutions (100 cc) of various concentrations of ammonium hydroxide for half an hour and then irradiated. Several lots were irradiated simultaneously with a control group treated only with distilled water which was used as a check on the X-ray dosage at each exposure. During the period of irradiation the seedlings were kept between filter papers, each moistened with the solution in which the seedlings had previously been immersed. After irradiation the seedlings were rinsed several times in distilled water and incubated for 3 hours. They were then fixed and the anaphases examined. Seedlings of *Vicia faba* (broad bean) were treated in a similar manner, except that they were left in the ammonium hydroxide solutions for 2 hours before irradiation to insure complete penetration since their roots are considerably thicker than those of *A. cepa* (root diameters approximately 0.8 to 1.0 mm and 0.2 to 0.3 mm respectively). Only 6 seedlings were placed in each beaker with 100 cc of solution. Throughout all of these experiments the temperature was kept at 22°-23°C. The results are shown graphically in Figs. 1 and 2, and in Tables I and III.

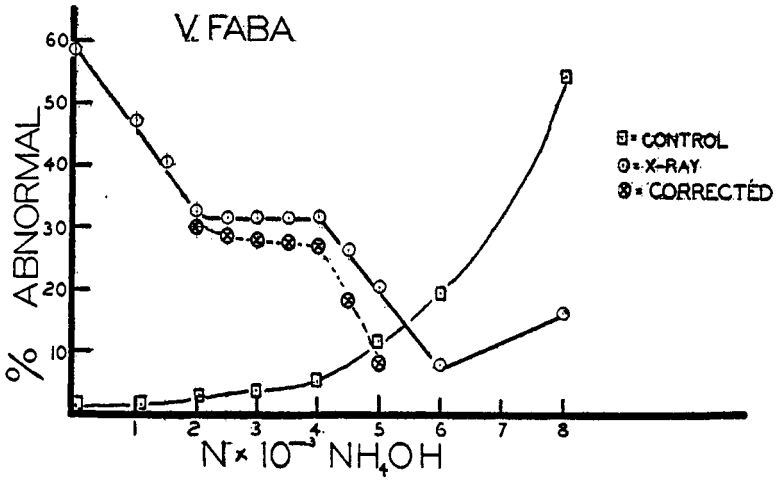

FIG. 1.
Vicia faba—100 Roentgens.

Per cent abnormal anaphases at 3 hours after irradiation with X-rays plotted against concentration of ammonium hydroxide in solutions in which the seedlings were immersed. Rectangles represent seedlings treated only with ammonium hydroxide and then incubated for 3 hours before examination. Open circles represent seedlings treated with ammonium and X-rays and then incubated. Crossed circles are obtained by subtracting the control values (rectangles) from the values after treatment with X-rays and ammonia (open circles) and represent the per cent abnormalities that may be attributed to the action of X-rays. Each point is based on counts of 150 to 1,000 anaphases except controls at concentrations 5.0 and 6.0 which are based on 68 and 97 anaphases respectively.

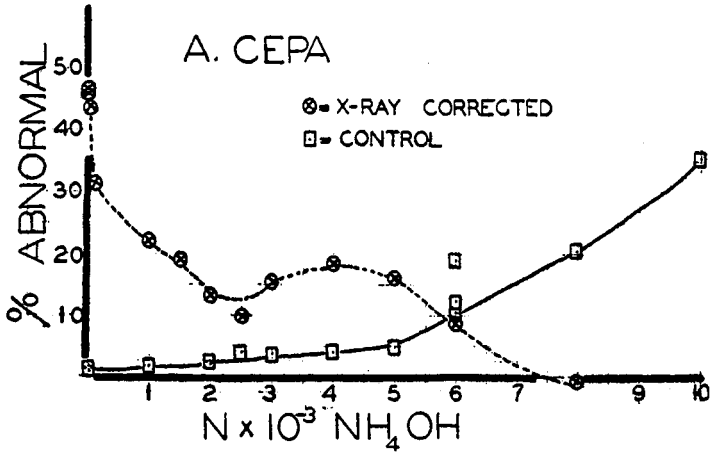

FIG. 2.
Allium cepa—80 Roentgens.

Symbols have the same significance as in Fig. 1. For simplicity the curve of open circles has been omitted. Each point is based on counts of 200 to 3,000 anaphases except control at concentration 4.0 which is based on 79.

It is evident from the figures that ammonia inhibits the formation of chromosome abnormalities by X-rays. For *V. faba,* with .002 N NH_4OH the reduction in sensitivity to X-rays amounts to a factor of approximately 2 and with .006 N to a factor greater than 60, since the corrected curve falls below 0% abnormalities at this point. Although the curve for X-rayed seedlings of *V. faba* shows a very definite plateau, the curve for seedlings treated only with ammonia gives no indication of one. With *A. cepa* the results are similar except that the curve for the X-rayed seedlings suggests that there may be a dip before the plateau is reached. More data are needed to determine whether the dip is real. The marked reduction in sensitivity by concentrations of ammonia which alone produce few or no abnormalities, and the different shapes of the X-ray and control curves indicate that the production of abnormalities by X-rays is due to their effect on a chromosomal system different from that which is altered by ammonia when it alone produces abnormalities. One effect of ammonia, and the only one at low concentrations, is to change the state of dissociation of the X-ray-sensitive materials in such a way as to render them less sensitive, presumably by changing the hydrogen ion concentration. The hypothesis that the X-ray-induced chromosome abnormalities are the result of the action of negative electrons on the positively charged groups of the dividing chromonemata is thus supported. Furthermore, since no abnormalities are produced by X-rays after treatment with .006 N NH_4OH for *V. faba* and .008 N for *A. cepa,* it would seem that at the intracellular pH corresponding to this concentration, the ionization of a large portion of the free positively charged groups is suppressed, leaving the separating chromonematic surfaces with a predominance of negative charges.* This finding would eliminate the protamines as the sensitive materials in these chromosomes, for their high content of arginine (87% of salmine, for example, is arginine) with a dissociation constant above pH 12 would require that the greater part of the X-ray-sensitive material still be positively charged and therefore capable of reacting at the high ammonia concentrations at which only a small proportion of the abnormalities may be attributed to X-rays.

Another interpretation might be given to the X-ray-ammonia

* At the higher concentrations of ammonia the per cent abnormal chromosomes is lower in the X-rayed seedlings of *V. faba* than in those treated with ammonia alone, which would suggest that X-rays in some way inhibit the tendency of ammonia to produce abnormalities. However, the phenomenon needs to be studied further before any very precise analysis of its nature can be made.

curves. It is possible that ammonia might arrest mitoses at late prophase or metaphase so that the cells observed later in anaphase may have been irradiated in one of these insensitive stages rather than at the onset of prophase. One might then expect an increase of metaphases or of all division stages at the time when the cells were fixed, 3 hours after irradiation. Instead, a marked decrease in the frequency of division stages was observed. Likewise dumbbell-shaped nuclei and binucleate cells in a relatively high frequency would be expected. Only very few were found (1 to 7 per slide with 30 roots of *A. cepa* at .001 N to .006 N), and these could be shown to be the result of multiple chromosome attachments and not the result of anaphase suppression. It seems most probable, therefore, that the anaphase cells counted had their abnormalities induced at the onset of the prophase as has been demonstrated for cells not treated with ammonia, and that the ammonia reduces sensitivity at this stage.

This conclusion is further supported by examining the percent abnormal anaphases at various intervals after immersion in ammonia. The roots examined at one-half hour after treatment with 0.0025 N NH_4OH showed the maximum number of abnormalities, which is approximately the time taken for the cell to develop from metaphase to middle or late anaphase (Table IV), indicating that the stage sensitive to the production of abnormalities by NH_4OH is the metaphase. Irradiation with X-rays produces a maximum number of abnormalities at 2½ to 3 hours after treatment, the sensitive stage being the onset of prophase, while the metaphase is quite insensitive. In producing abnormalities in metaphase, therefore, ammonia must act on quite a different chromosomal system than X-rays and also in reducing the sensitivity to X-rays at onset of prophase the ammonia must in some way be acting on the X-ray sensitive portion of the chromonema.

Ammonia has been known to produce dumbbell-shaped nuclei and

TABLE I.

A. cepa—Experiments 130, 131. X-ray—80 r. in air. Calculated Dose Received when in Chamber—75 r.

a volume % CO_2	b abnormal	c total	d % abnormal
air only	400	856	46.7
11	550	1237	44.5
20	238	565	42.2
40	324	765	42.5
60	208	449	46.4
80	206	443	46.5

TABLE II.
Summary NH_4OH—X-ray. Experiments 146, 149, 150.
V. faba—100 r. (approximate, see note below)*

a		b	c	d	e	f
Conc. NH_4OH N $\times 10^{-3}$	Exp.	Abnormal	Total	% abnormal	% abnormal corrected for dose	e minus control d. X-ray effect only
0	146	417	711	58.7	58.7	58.2
0	149	579	1043	55.5	58.7	58.3
0	150	264	419	63.1	58.7	58.7
1.0	146	207	439	47.2	47.2	46.5
1.5	150	461	1061	43.5	40.5	39.0
2.0	146	151	465	32.5	32.5	30.0
2.5	150	247	726	34.0	31.6	28.7
3.0	149	68	228	29.8	31.6	27.7
3.5	149	48	161	29.8	31.6	27.6
4.0	146	58	182	32.0	32.0	27.6
4.5	149	88	351	25.0	26.5	19.0
5.0	149	40	207	19.3	20.4	8.6
6.0	146	18	227	8.0	8.0	—11.6
8.0	146	38	232	16.5	16.5	—38.1
		Controls—No X-ray.				
0	146	3	625	0.5		
0	149	6	1097	0.4		
0	150	—	—	—		
1.0		5	643	0.7		
2.0		11	457	2.5		
3.0		14	358	3.9		
4.0		9	203	4.4		
5.0		8	68	11.8		
6.0		29	147	19.6		
8.0		52	97	54.6		

*In experiments 149 and 150 there was a small but definite and uncontrollable fluctuation in voltage which made it impossible to get the dose exactly from dosimeter readings. The per cent abnormalities from seedlings treated only with water at each exposure has been used to obtain a correction factor to the X-ray dose value of experiment 146. The 58% abnormal for this experiment corresponds to 100 r. as determined from the survival curve by the method previously described.[2] In experiment 146 it was possible also to hold the voltage steady and the dosimeter readings gave the dose as 100 r. Voltage fluctuations did not complicate results in any of the other experiments.

binucleate cells.[3, 4] Kuwada and Nakamura[5] treated dividing plant cells with ammonia fumes and observed swelling of the chromosomes and "unraveling" of chromonemata resulting in a condition resembling a resting stage nucleus. A special effort was made while carrying out these experiments to find the swelling and "unraveling" described. At no stage after treatment with ammonia was there found to be a generalized swelling or "unraveling", although at ½ hour after treatment about half the anaphase cells had one or more attached chromosomes. When several chromosomes become

3 Mainx, F., *Zool. Jahrb. Abt. f. Allg. Zool. u. Physiol. der Tiere,* 1924, **41**, 553.

4 Rosenfeld, M., *Arch. exp. Zellf.*, 1933, **14**, 1.

5 Kuwada, Y., and Nakamura, T., *Cytologia,* 1934, **5**, 244.

TABLE III.
Summary—NH_4OH—X-ray Experiments 127, 132, 133, 148.
A. cepa—80 r.

Conc. NH_4OH N × 10^{-3}	Abnormal	Total	% abnormal	d minus control d X-ray effect only
0 (127)	383	799	48.0	46.3
0 (157)	197	423	46.5	45.7
0.01	271	597	45.4	43.7
0.1	139	422	33.0	31.3
1.0	176	743	23.7	22.1
1.5	61	291	20.9	18.9
2.0	40	257	15.6	13.1
2.5	28	203	13.8	9.8
3.0	50	261	19.2	15.5
4.0	59	264	22.3	18.3
5.0	40	192	21.0	16.1
6.0	76	394	19.3	8.8
8.0	107	543	19.7	—0.8
10.0	125	435	28.7	—6.9
	Controls—No X-ray.			
0 (157)	66	3953	1.7	
0 (162)	12	1530	0.8	
1.0	15	956	1.6	
2.0	6	238	2.5	
2.0	13	655	2.0	
2.5	28	696	4.0	
3.0	23	628	3.7	
4.0	3	79	3.8	
5.0	34	699	4.9	
6.0	45	248	18.0	
6.0	34	325	10.5	
6.0	34	274	12.4	
8.0	41	200	20.5	
10.0	242	681	35.6	

TABLE IV.
Anaphase Chromosome Abnormalities as a Function of Time After Treatment with 0.0025 N NH_4OH

Time after treatment in hours	0	½	1	2	3	4	5
Total anaphases	888	1107	588	547	371	473	426
% normal	68.0	51.9	87.0	92.4	95.7	95.5	96.0

so attached, the daughter anaphase groups fail to separate as sometimes happens to cells receiving heavy doses of X-rays. The observations of Kuwada and Nakamura may be interpreted, therefore, not as a direct swelling and "unraveling" produced by ammonia, but as the usual sequence of changes observed in telophase and resting stage of chromosome groups whose separation has been prevented.

Zirkle[6] has studied the relation of varying concentrations of ammonia, carbon dioxide and hydrogen sulfide on X-ray inhibition of fern spore germination and the first cell division of that spore. Curves for the effects of acids and bases alone are not given, but

[6] Zirkle, R., *Am. J. Roent. and Radium Therapy*, 1936, **35**, 1.

curves for the relative X-ray effects of the chemically treated spores as compared with irradiated, but non-treated spores are presented. Ammonia appears to inhibit the X-ray action at low concentrations, the effect reaching a maximum at about 0.003 N and then at higher concentrations, 0.008 N, markedly increases the X-ray sensitivity. The acids, on the other hand, increase sensitivity at low concentrations and decrease it at high concentrations. It is possible that the fern spore results may be related to the observations on chromosomes here presented, but there are not enough data available for the observations on the spores to show any direct correlations. In interpreting his results Zirkle assumes one or more sensitive proteins associated with the cell function studied which is normally in a medium on the alkaline side of its isoelectric point. Added acids increase sensitivity until the isoelectric point is reached and then decrease it. Although ammonia does decrease sensitivity as expected on his hypothesis, there is no adequate explanation for the observed turning point at 0.003 N, for if toxicity is to be invoked as an explanation here, it might also be used for the turning point on the acid side which is presumed to represent the isoelectric point.

The pH range included by distilled water solutions of 0.001 N to 0.01 N NH_4OH is approximately 10.27 to 10.77.[7] The absolute value of the intracellular pH is not known. However, if the internal pH is reduced by the interaction of ammonia ions with intracellular anions, the plateau of the X-ray curve might conceivably correspond with the steep portion of the dissociation curve of ammonia, where a relatively large change in ammonia normality is needed to alter appreciably the percent dissociation and hence the percent reduction in sensitivity of chromosomes to X-rays. However, if ammonia dissociation were the only factor involved one would expect a similar plateau in the ammonia control curve. There is no indication of one with *V. faba* or with *A. cepa*.

The X-ray curve for *V. faba* shows an interesting resemblance to protein titration curves and it may be possible to analyze it further in terms of the constituent amino acids of the proteins involved. As previously indicated the guanidine group of arginine with a pK of 12.5 would not be involved in the reaction while lysine with pK values 8.95 and 10.5 and cysteine with pK 8.2 and 10.3 might very well be.† Thus the chromosome response to ammonia and X-rays

[7] Michaelis, L., *Hydrogen Ion Concentration*, Baltimore, 1926, trans. by A. Perlzweig.

† Dissociation constants taken from 8.

[8] Cohn, Edwin J., *Ergebnisse der Physiologie*, 1931, **33**, 781.

leads to the conclusion that the sensitive proteins are probably histones, and suggests the possibility of identifying those of its amino acid constituents which take part in the reaction. Whether these X-ray-sensitive amino acids can be more specifically and perhaps quantitatively ascertained remains for future experimentation to determine.

Summary. Treatment with ammonia reduces the sensitivity of chromosomes to X-rays. Curves showing the decrease in sensitivity as a function of ammonia concentration are presented. The results are taken as evidence in support of the hypothesis, previously advanced, that the sensitivity of chromosomes to X-rays at the onset of prophase may be attributed to the presence of positive charges on the separating surfaces of the dividing chromonema. These separating surfaces are probably made up of proteins of the histone type.

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A Corpus Luteum-Stimulating Substance in the Rat Placenta.*

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In the majority of mammals the length of the pregnancy cycle exceeds that of the normal estrous or pseudopregnant cycles, and this prolongation is due in part, to a sustained function of the corpora lutea. The presence of foetal elements is in some way responsible for this continued activity of the maternal ovary.

The function of the corpora lutea of the rat during the first half of pregnancy is under the control of the hypophysis and the mechanism involved is apparently the same as that in pseudopregnancy. Luteal function is essential throughout pregnancy, however, as shown by the fact that bilateral öophorectomy invariably terminates gestation.[1-4] Removal of the pituitary gland during the first half of pregnancy is likewise followed by death and resorption of the foe-

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† Rockefeller Foundation Fellow in the Natural Sciences.

1 Harris, R. G., and Pfiffner, S. S., *Anat. Rec.*, 1929, **44**, 205.

2 Johnson, G. E., and Challans, J. C., *Anat. Rec.*, 1930, **47**, 300.

3 Nelson, W. O., and Haterius, H. O., *Physiol. Zool.*, 1930, **3**, 231.

4 Hain, A. M., *Quart. J. Exp. Physiol.*, 1934, **24**, 101.