The Action of Mustard Gas on Ox Cornea Collagen

By ANTOINETTE PIRIE, *Nuffield Laboratory of Ophthalmology, Oxford*

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The reaction between ox corneal collagen and β : β' -dichlorodiethylsulphide (mustard gas, referred to in the text as *H*), has been studied during work on the effect of various chemical warfare agents on the eye. The aim of the investigation was to establish whether the two substances combined, and, if so, what were the properties of the compound formed, what groups in the protein molecule were involved and whether the compound could be broken up and the normal protein restored. The experiments have shown that *H* combines with collagen under conditions likely to obtain in the eye and that a change in the physical and chemical properties of the treated collagen can be demonstrated.

H causes lesions of the cornea in both man and animals. The histology of small avascular lesions, made by placing small drops of *H* directly on the centre of the rabbit's cornea, has been described by Pullinger & Mann (1943). All cells—the epithelium, the corneal corpuscles and the endothelium—in the path of the liquid are destroyed and subsequently disappear. The collagen fibrils become separated by oedema fluid but do not themselves swell or dissolve, nor are they sloughed out. The collagen fibrils of the cornea make up 97% of the dry weight of the tissue so that a reaction between collagen and *H* to give a product with different properties might be of importance in determining the type of injury. This also holds true for injury of the skin by *H* as collagen is the main protein of the cutis.

In all the experiments reported, fresh collagen that had not been dried at any stage has been used. This was considered important because collagen loses some of its power to swell after being even partly dried. The reaction between *H* and collagen was studied in a neutral solution of 0.9% NaCl at 30° in order to simulate physiological conditions. The amount of *H* which had combined was estimated by determining the rise in sulphur content of the collagen after treatment, and tests of solubility, enzyme digestibility, swelling and stability, etc., were made on both normal and treated collagen preparations and titration curves were made in each case.

METHODS AND RESULTS

Preparation of collagen

Collagen was prepared by the method of Bergmann & Stein (1939). Fresh ox corneas were minced with scissors

and suspended in 10% NaCl at 0°. After 24 hr. the corneas were strained off through muslin and minced in a small latapie mincer. The fresh corneas were too tough to go through this mincer, but after the above preliminary extraction the fragments were swollen and were more easily cut. The minced tissue was replaced in fresh 10% saline at 0°. Extraction with saline was continued for a week, with a change of fluid each day, about 5 l. in all being used to extract 100 corneas. The tissue was then extracted with $M/15-Na_2HPO_4$ at 0° for 3–4 days, the fluid being changed each day, and then with distilled water, until the extract was free from chloride. The wet corneal residue was stored under ether at 0°. This residue was taken to be collagen. There is little elastin in cornea and the remaining corneal constituents are soluble in the extracting fluids. A heavy precipitate of mucin came down on acidifying the early extraction fluids. The residual collagen was much swollen and translucent. After removing as much water as possible by hand pressing between filter paper, the dry weight was found to be 11–14% of the wet weight.

Estimation of sulphur. Known weights of 5–30 mg. of collagen dried over P_2O_5 were incinerated with a perchloric-nitric-copper mixture described by Pirie (1932) and the total sulphur in the incinerate was estimated by the colorimetric method of Cuthbertson & Tompsett (1931). Duplicate incinerations and estimations were done and it was found necessary to do a blank estimation each day.

Reaction of corneal collagen with H

A thick suspension of collagen in 0.9% NaCl was made in a small beaker. Neutral red was added as an internal indicator and the suspension was kept mechanically stirred at 30° in a water-bath. Liquid *H*, 0.05–0.2 ml. was then added and as soon as the indicator changed colour 0.1N-NaOH was added from a burette to bring the suspension again to neutrality. The reaction was followed until at least 80% of the added *H* had been hydrolyzed, as measured by the total NaOH that had been run in. The collagen was then washed several times with water on the centrifuge until the supernatant fluid was free from chlorine. It was then washed by shaking with several changes of ether to remove residual *H* and stored at 0° under ether. Before analysis, samples were dried *in vacuo* over P_2O_5 and most were then extracted, either with ether or acetone, to remove the last traces of free *H*, or its hydrolysis products.

The rate of hydrolysis of *H* in water depends on the degree of dispersion of the *H*. If it is added as an ethanolic solution hydrolysis is very rapid, but in a purely aqueous medium it is slow and depends on the rate of stirring. Peters & Walker (1923) have shown that NaCl retards hydrolysis. The rate was increased in the presence of collagen but it is difficult to judge whether this was due to

reaction of *H* and collagen or to mechanical break-up of the globules of insoluble *H* by the thick protein suspension. The time for 50% hydrolysis of 0.04 ml. *H* was 180 min. in water and 270 min. in 0.9% NaCl, while 0.05 ml. *H* was 50% hydrolyzed in 75 min. in a thick suspension of collagen in 0.9% NaCl. The reaction was followed by titrating the liberated HCl with 0.1N-NaOH. The suspensions were continuously stirred at 30°.

Table 1 gives the total S contents of different preparations of ox cornea collagen before and after reaction with *H*. Mörrner (1899) gives 0.2% S in corneal collagen and Bergmann & Stein (1939) give 0.22% S in tendon collagen.

Table 1. *Sulphur content of ox cornea collagen before and after treatment with H*

Each figure represents the mean of duplicate or triplicate estimations, except in sample no. 6.

No.	Sulphur (%)	
	Collagen	<i>H</i> collagen
1	0.18	0.63
2	0.18	0.43
3	0.26	0.90
4	0.33	1.0
5	0.20	0.69
6*	0.19	0.46

* Collagen from rat tail tendon; single estimation in each case through lack of material.

Table 1 shows that in every case the total S content has risen after the treatment with *H* but that the rise varies from one preparation to another. It is possible that none of the preparations was saturated with *H*. In preparations 1 and 2, 0.1 ml. *H* was added to 5–10 g. wet collagen and in other preparations which have a higher final S content, 0.2 ml. *H* was used with approximately the same amount of protein. No attempt was made to reach saturation of collagen with *H* as this would probably not occur under natural conditions.

Comparison of the properties of normal and *H* treated collagen

(a) *Swelling*. A characteristic property of collagen is the degree of swelling in acids and alkalis of different strength. Jordan Lloyd (1933) plotted the swelling curves of fresh skin collagen between pH 1 and 12 and found a maximum between pH 2–3. The swelling of normal ox cornea collagen was measured and compared with that of ox cornea collagen after treatment with *H*.

The fragments of collagen and *H*-treated collagen were pressed by hand between filter papers to remove as much water as possible and 0.9 g. samples were weighed into a series of centrifuge tubes. Water, acid or alkali of known strength (10 ml.) was added and the tubes immediately stoppered. After 48 hr. on the bench at 15° with occasional shaking, the supernatant fluid from each tube was pipetted off and its pH determined either by titration or with the glass electrode. The collagen residues were washed once on the centrifuge with water and allowed to drain as com-

pletely as possible. The wet weight and the dry weight of each sample was then determined and the degree of swelling taken to be wet weight $\times$ 100/dry weight.

Fig. 1. Swelling curves of normal ox cornea collagen and *H*-treated ox cornea collagen. +—+—+ normal; o—o—o *H*-treated.

Fig. 1 shows that *H*-treated collagen does not swell much either in acid or in alkali. The suppression of swelling is greatest in acid as it is here that the normal protein is most swollen. In neutral solution *H*-treated collagen and normal collagen have their zones of minimum swelling and here both are swollen to about the same extent. A difference in appearance between normal and *H*-treated collagen is discernible at neutrality but becomes much more obvious in acid solution where the normal protein is bluish and translucent and *H* collagen is white and opaque.

(b) *Solubility*. Normal collagen is dissolved and forms gelatin when boiled in water. Table 2 shows the proportion

Table 2. *Conversion of collagen and H collagen to gelatin by treatment with boiling water*

No.	Collagen		<i>H</i> collagen	
	Soluble (%)	Insoluble (%)	Soluble (%)	Insoluble (%)
1	82	18	10	90
2	—	—	5	95
3	83	17	10	90
4	69	31	4	96

S content of soluble and insoluble fractions

No.	S content of collagen (%)			S content of <i>H</i> collagen (%)		
	Original	Soluble	Insoluble	Original	Soluble	Insoluble
1	0.26	0.09	0.92	0.93	1.07	0.77
2	—	—	—	0.93	0.67	0.94

of collagen and of *H*-treated collagen converted to gelatin by heating in a boiling water-bath for 8 hr. at pH 4.0 under a simple condenser. The table gives also the S contents of

the soluble and insoluble fractions. The insoluble residues were separated by centrifugation and in preparation 1 both residues and supernatant fluids were frozen and dried over P_2O_5 without further treatment. In preparation 2 the supernatant fluid from *H* collagen was precipitated with ethanol before drying in order to remove any *H* or breakdown products which might have been split off during heating.

H-treated collagen is practically insoluble and judging from the S contents of the soluble and insoluble fractions it is stable under these conditions. It is interesting that most of the S in the normal collagen is contained in the small insoluble fraction.

(c) *Enzyme digestibility.* The effect of different proteolytic enzymes was tested by incubating them with the finely chopped collagens in a concentration of 5–20 mg./ml. Samples were taken in a 1.0 ml. all-glass syringe whose tip had been cut off to give a wide opening. If the suspensions were shaken during sampling, fairly good duplicate samples could be taken. These were centrifuged, the insoluble protein was washed once on the centrifuge, dried at 90° and weighed. The loss in weight during incubation with the enzyme was taken as a measure of digestion. Controls showed that no solution of collagen or of *H*-treated collagen took place at the pH used for digestion by papain or cathepsin, but both activated enzymes partly dissolved collagen in one or two of the experiments though not in all. Normal collagen went into solution rather more readily than *H* collagen but the difference is probably insignificant owing to the difficulty of sampling. Some normal collagen went into solution at pH 1.8, the optimum pH for pepsin, but this did not obscure the effect of the enzyme which caused almost complete solution of normal collagen. *H*-treated collagen, in striking contrast to the normal protein, was not digested by pepsin. The activity of the enzymes was tested by the usual methods.

Table 3. *Effect of proteolytic enzymes on collagen and H collagen*

Enzyme	pH	Time	Loss in weight during digestion (%)	
			Collagen	<i>H</i> collagen
Trypsin	7.8	48 hr.	0	0
Papain	5.2	24 hr.	12	0
Papain		24 hr.	41	24
Papain		24 hr.	0	10
Papain		3 weeks	44	12
Cathepsin	5.2	24 hr.	38	12
Cathepsin		48 hr.	0	0
Cathepsin		10 days	0	0
Pepsin	1.8	24 hr.	95	18
Pepsin		24 hr.	95	0
Pepsin		24 hr.	90	0
Pepsin		24 hr.	99	18
Pepsin		24 hr.	87	0
Pepsin	4.0	8 days	0	0

Percentage loss in weight of 1.0 ml. samples after digestion at 37°. The enzymes used were: trypsin (0.2%), papain (0.1%) activated with HCN, and pepsin (0.1%) (all British Drug Houses Ltd.). Liver cathepsin (Anson, 1937) was activated by cysteine (Fruton, Irving & Bergmann, 1941).

(d) *Stability of H collagen in acid and alkali.* The higher S content, the resistance to pepsin and the insolubility in boiling water at pH 4 of *H*-treated collagen were used as criteria in attempts to detach the *H* residues from the collagen molecule leaving the protein in its original condition. If the reaction between *H* and collagen could be reversed these differences should disappear. In brief, *H* collagen was found to be unstable in dilute alkali where it lost a large part of its extra S, became readily digestible by pepsin and soluble on boiling. Samples of collagen were suspended in 0.02% KOH and left for 24 hr. at room temperature. This seemed to cause some swelling of the *H* collagen but no gross change occurred in either preparation. Merrill (1924) studied the effect of alkali on the hydrolysis of skin collagen and found that none occurred, as judged by absence of N in the supernatant, after 24 hr. at 15° and pH 13.

Table 4. *Effect of alkali on collagen and H collagen*

100 mg. normal and *H* collagen were suspended in 10 ml. 0.02% KOH and left for 24 hr. The sediments were centrifuged down, washed several times and then dried in the frozen state.

(a) Effect on S content					
Percentage S before KOH		Percentage S after KOH		Change in percentage S	
Collagen	<i>H</i> collagen	Collagen	<i>H</i> collagen	Collagen	<i>H</i> collagen
0.43	0.64	0.29	0.36	-0.14	-0.28
0.32	0.98	—	0.35	—	-0.63
(b) Effect on pepsin digestibility					
Digestibility (%)					
Before KOH			After KOH		
Collagen	<i>H</i> collagen		Collagen	<i>H</i> collagen	
95	18		95	95	

Tests of the pepsin digestibility of *H* collagen held for 24 hr. at 37° in buffers of different pH showed that after incubation at pH 4.7, *H* collagen became 18% digestible, after incubation at pH 7.0 it became 34–45% digestible and after incubation at pH 9.5 it became 99% digestible. A test of the convertibility of *H* collagen to gelatin at different pH ran parallel to the test of pepsin digestibility. At pH 9.5, 85% went into solution but at lower pH little dissolved. The alkaline extracts from *H* collagen gave a positive test for thiodiglycol with selenium dioxide.

Mode of combination of H with collagen

The experiments already reported have shown that combination of collagen with *H* alters its properties considerably. The question arises whether these alterations can give any clue to the mode of linkage of *H* in the treated collagen molecule. The evidence obtainable from the reaction between collagen and other substances, such as formaldehyde, whose point of attachment is known, is meagre. For example Ewald (1919) found that formol-treated collagen was insoluble and was not digested by pepsin, and Hopkins & Wormald (1933) found that gelatin became insoluble after treatment with phenyl isocyanate. In both these cases the free NH_2 groups were blocked by the reagent, and by analogy one might suppose that *H* also has combined with

the free NH_2 groups to give insoluble and non-digestible *H* collagen. On the other hand, the alkali lability of *H* collagen suggests that the reaction is an esterification of the carboxyl groups. Blackburn, Carter & Phillips (1941) found that methylated wool which contained mainly ester methoxyl groups was alkali-labile and gave up its methyl groups at room temperature and pH 9.5.

Further evidence as to the method of linkage of *H* was sought by determination of the titration curve of the normal and treated proteins and a comparison of their staining reactions with acid and basic dyes. The method of determination of the titration curves was essentially the same as the method of determination of the swelling curves with the modification that the acids and alkalis were made up in 0.1M-KCl as this is thought to give a more uniform penetration of the acid or base into the collagen gel. The preparation of *H* collagen used had a rather low total S content of 0.5%. Normal collagen has 0.2% S, so that 0.30% S in the *H* collagen may have come from *H*. This is a small amount to make a detectable difference in the titration curve and, as expected, the titration curves for normal and *H* collagen were very much alike. *H* collagen appeared to combine with slightly less acid and more alkali than normal collagen but the difference was too small for much reliance to be put on the result. Tests of the staining reactions of normal and *H*-treated collagen with acid and basic dyes showed that, compared with the normal protein, *H* collagen stained less intensely with basic dyes such as methylene blue and methyl violet, and more intensely with acid dyes such as eosin and acid fuchsin. The difference in staining was very clear if small fragments of the collagen were suspended in the dye solutions for a few minutes and then washed once or twice with water. These tests indicate that there was a reduction in the acid groups of collagen as a result of *H* treatment.

A further analysis of an acid hydrolysate of *H* collagen to determine whether any *H* combined with the free NH_2 groups of the molecule has been carried out by Syngé & Gordon, using the chromatographic method elaborated by Gordon, Martin & Syngé (1943). Acids equally or more readily extractable by the organic solvent than acetylalanine would thus be determined. The normal collagen used for analysis had 0.21% S and the *H* collagen 0.69%. Syngé & Gordon state no qualitative differences (anomalous bands, etc.) between the normal and *H* collagen were observed during the analysis, and there were no quantitative differences exceeding the experimental error. The procedure would be expected to reveal any reaction of *H* with free NH_2 groups, etc., giving rise to *N*-substituted acid-stable compounds of amino-acids; such compounds after hydrolysis should give acid acetyl derivatives readily extracted by organic solvents. (The main free NH_2 groups in collagen are probably ϵ -amino-groups of lysine residues according to Gurin & Clark (1934).) Thus, while the present work, being negative in outcome, must be interpreted with caution, it suggests that *N* substitution of collagen does not occur during reaction with *H* and that the main reaction is probably an *O* substitution.

On the whole, the evidence is in favour of the view that *H* combines with the free carboxyl groups of collagen although, from the properties of the formol-treated collagen described by Ewald (1919), it would seem that reduction in NH_2 groups also leads to greater insolubility and less ready digestibility by pepsin.

Occurrence of H collagen in vivo

The preparation of a compound of *H* with collagen does not necessarily mean that such combination takes place when *H* burns either the eye or the skin. Some attempts to assess the importance of *H* collagen *in vivo* were made by investigating, first, whether *H* collagen was formed when *H* reacted with whole chopped cornea and, second, whether the tissue reaction to implanted *H* collagen bore any relation to the tissue reaction to an *H* burn of the skin.

Reaction of H with chopped cornea. Fresh chopped ox cornea was suspended in saline and allowed to react with *H* in the usual way. After this treatment 10% NaCl or $\text{M}/15\text{-Na}_2\text{HPO}_4$ did not extract any corneal mucin, although these solvents remove all mucin from normal cornea. Berenblum (1940) found that after treating skin with *H* very little nucleoprotein could be extracted from it and he considered that the nucleoprotein had been precipitated by the *H* in the skin. The insolubility of the mucin from the cornea may either be due to a change in the permeability of the collagen gel, in whose interstices the mucin is normally held, or it may be due to a reaction between *H* and corneal mucin. No evidence for such a reaction has been obtained and it was found that the mucin could be successfully removed from *H*-treated cornea by digestion with pepsin. Digestion was carried out for 10 days at 37° with several changes of enzyme solution. After this time, as there seemed no further change in the insoluble residue, it was centrifuged, washed repeatedly with water and its properties compared with those of *H* collagen. A dried sample, after ether extraction, gave total S, 0.74% and in general the properties were similar to those of other preparations of *H* collagen. This result shows that *H* combines with collagen in the chopped tissue as well as in a suspension of the purified protein.

Tissue reaction to implantation of normal and H collagen. Implants of normal and of *H* collagen were made in two rabbits. This experiment was done in collaboration with Dr B. D. Pullinger and I am indebted to her for the histological description of the lesions and the tissue reaction to the implants. The rabbits were laboratory stock (crossed Dutch). Two large areas, one on each side of the mid-ventral line, were shaved. The following day the animals were anaesthetized and four small slits made in each of the shaved areas. About 10 mg. (dry) of normal collagen was implanted subcutaneously through each of the four slits on one side of the animal and about 10 mg. (dry) of *H* collagen implanted in the same way on the other side of the animal. The implantations were done with a metal spatula and the small lumps of protein were pushed away from the slit in the skin. The slits were then stitched up. The animals recovered quickly. At first the animals were examined and the appearance of the reactions noted each day; later, weekly examinations were made. At the end of the first and nearly all subsequent weeks a piece of normal and a piece of *H* collagen were removed from one or other of the animals alternately. The animal was anaesthetized and the protein fragment attached to the surrounding skin was cut out and dropped immediately into Zenker's fixative. The loose edges of skin were then stitched together. The sterility of samples of normal and of *H* collagen was tested by aerobic incubation in broth. No turbidity appeared in 48 hr. Sections were also made of the fragments embedded in agar and stained with Heidenhain's haematoxylin and

other bacterial stains. A few colonies of a diphtheroid bacillus were found.

The following is a summary of the histological reactions of the various normal and *H* collagen preparations.

The comparison lies between the tissue reaction to free normal collagen and to free *H* collagen, prepared by peptic digestion of *H*-treated cornea. The differences are quantitative rather than qualitative. They are visible in both macroscopic and microscopic specimens. Swelling and hyperaemia are far greater in the *H*-treated than in the normal specimens. The swelling increases up to 10 days then slowly decreases over a period of several weeks. The microscopic differences are so great that for 5-6 weeks it is possible to distinguish and name the specimens correctly even when they are unlabelled. The implanted masses of *H*-treated and normal collagen are encapsulated within a week by a fibrocellular capsule which soon becomes vascular. The cells of the capsule are mainly fibrocytes, granular leucocytes and lymphocytes. The normal collagen lies in the capsule surrounded by a few granular leucocytes and macrophages, but in the case of the *H* collagen it is surrounded by enormous numbers of these cells. It is this difference in the number of invading cells which enables one to distinguish the two specimens at any time before the sixth week. Many of these cells are ill-preserved at and after 3 weeks owing to karyolysis and to pyknosis of nuclei. From the fourth week onwards, large numbers of a particular kind of macrophage and of lymphocytes (sometimes in nodular form with accompanying reticulum cells) appear within the capsule. These macrophages have abundant foamy cytoplasm containing material which is stainable with van Gieson's solution and with Mallory's aniline blue. At 5 weeks all the free normal collagen, and at 8 weeks all the free *H* collagen has disappeared except for the stainable material, probably ingested collagen, in the macrophages. (No observations were made on the *H* collagen at 6 and 7 weeks; one specimen was lost through dispersal in the loose tissue of the groin; another ulcerated out through a stitch abscess.)

The features worthy of notice in these lesions are: (1) the slow disappearance of normal and *H* collagen (the *H* collagen being slightly the slower of the two); (2) the more intense inflammatory reaction to *H* collagen as shown by greater swelling, hyperaemia and cellular migration.

The second feature indicates that while both normal and *H* collagen cause an inflammatory reaction, the *H* collagen is considerably more poisonous than the normal collagen. The presence of polymorphs points also to a certain degree of solubility of the two collagens. There is probably some chemotactic and sensitizing action. These leucocytes appear to be unable to digest either collagen, yet when the stimulus for their presence has ceased and they have died *in situ*, the collagen becomes ingested by macrophages.

DISCUSSION

The suggestion has been made (Pullinger, 1946) and evidence presented to support it, that the action of *H* on the skin is to cause coagulation necrosis. The outstanding difference between this and other forms of necrosis is the relative resistance of the dead product to solution by tissue cells or fluids. Thus it can lie *in situ* for considerable periods of time. *H* collagen *in vitro* is relatively resistant to solution by

enzyme action and according to the experiments recorded it is also insoluble *in vivo* after subcutaneous implantation.

The evidence from the experiments on tissue reaction to implantation is in favour of the view that the insolubility of tissue 'burned' by *H* may in part at least be due to the presence of a compound of *H* and collagen. That such a compound of *H* with collagen can be formed possibly by reaction of *H* with the carboxyl groups is shown by the *in vitro* experiments here recorded.

Evidence that *H* remains fixed in the cornea has also been obtained through the use of *H*-containing radioactive sulphur. Hamilton & Axelbrod (1943), describing the detection of radioactive *H* in tissue sections, showed that considerable amounts of *H* were detectable in rabbit cornea 7 days after the cornea had been exposed to *H* vapour. The *H* was distributed throughout the whole thickness of the cornea, showing that it had combined with some insoluble corneal constituent. Other substances, such as benzoylchloride and *o*- and *p*-chlorobenzylchlorides which have been shown by Medveczky & Uhrovits (1931) and by Landsteiner & Jacobs (1936) to form compounds with proteins also cause coagulation necrosis of mouse skin (Pullinger, 1946), although the tissue is not so deeply affected as it is by *H*.

There is, of course, ample evidence that there are many other ways in which *H* may damage the skin and the cornea, e.g. by inhibition of specific enzymes as described by Dixon & Needham (1946). It has been the purpose of this paper to define one possible reaction between *H* and collagen and to determine whether the properties of the reaction product fit it to play a part in the pathology of the *H* lesion.

SUMMARY

1. The reaction between ox corneal collagen and β : β' -dichlorodiethylsulphide (*H*) has been studied.
2. *H* combines with collagen to give a product with a higher S content. This compound, unlike normal collagen, swells only slightly in acid solution, is resistant to pepsin digestion, and is insoluble when boiled in dilute acid.
3. The mode of combination of *H* with collagen is discussed.
4. The tissue reaction to implanted *H* collagen is described and it is concluded that some of the characteristics of an *H* 'burn' of the skin or eye may be due to the presence of *H* collagen in the injured tissue.

This work was done between 1940 and 1944, partly at the Laboratories of the Imperial Cancer Research Fund, Mill Hill, and partly at Oxford. I wish to thank the Director of Scientific Research (Defence), Ministry of Supply, for permission to publish the results. It is also a pleasure to thank Dr W. E. Gye for his help and interest, Dr Pullinger for carrying out the experiments on implantation of collagen, and Dr R. L. M. Synge and Dr H. Gordon for analyzing *H* collagen.

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The Determination of Dienoestrol

By M. B. SAHASRABUDHE (Government of India Scholar) AND
 A. E. WILDER SMITH (Countess of Lisburne Memorial Fellow),
Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1

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Since the clinical and veterinary use of synthetic oestrogens has become so general it is important to devise a simple routine method for the determination of these substances, particularly in biological samples. It would also be an advantage to possess a method applicable at the same time to pharmacological preparations.

Many attempts have been made in this direction, references to which are given by Malpress (1945) but it is probably fair to state that none is applicable to samples of synthetic oestrogens containing biological contaminants. The colouring matter in urines for example, interferes with colorimetric methods, e.g. the Dingemanse (1939) antimony pentachloride method (Warren & Goulding, 1945) and the presence of other phenols interferes with methods dependent on nitration (Malpress, 1945). No method has yet been evolved which will overcome these difficulties by quantitative separation of the synthetic oestrogen or a derivative.

The present communication describes a method of separating a derivative of dienoestrol in about 70 % efficiency from most other contaminants likely to be found in biological samples. It makes use of the facts that dienoestrol is a diene and reacts with maleic anhydride and that the resultant adduct is soluble in aqueous sodium bicarbonate solution,

(Wilder Smith, 1946). The method may be represented diagrammatically as follows: