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EXPERIMENTAL LIVER CIRRHOSIS IN RATS PRODUCED BY PROLONGED SUBCUTANEOUS ADMINISTRATION OF SOLUTIONS OF TANNIC ACID.

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IT has been shown that a cirrhosis, resembling human cirrhosis of the Laennec type, can be produced in experimental animals by abnormal diets (Glynn, Himsworth and Lindan, 1948; Wahi, 1949). On the other hand, Cameron and Karunaratne (1936) and Steinberg and Martin (1946) have shown that the experimental cirrhosis of the liver may, in its reversible stage, end in complete anatomical recovery. These facts gave rise to the hope that appropriate dietetic treatment might be successful in human cirrhosis. It is now well known that the ingestion of a suitable diet and lipotropic substances frequently exerts a favourable effect on human cirrhosis, which can be confirmed histologically. Unfortunately, the same therapy may fail completely in other, occasionally not advanced cases. These facts indicate, in our opinion, that cirrhosis of the liver is not invariably a deficiency disease, but that other factors can play a part in its aetiology.

One of us (B. K.) assumed as early as 1942, independently of English (Cameron, Milton and Allen, 1943) and American authors (Wells, Humphrey and Coll, 1942), on the basis of the changes found in the liver of persons who had suffered burns and been treated with tannic acid, that tannic acid had a hepatotoxic effect. He administered tannic acid solution parenterally to experimental animals and found central acinar necrosis in the livers of those animals which died after 24 to 26 hours. Vascular phenomena simulating the pattern of a serous hepatitis may be observed before the development of the parenchymal lesion (Korpássy, 1949).

The hepatotoxic effect of tannic acid having been proved, the question of what effect the protracted treatment with sublethal doses of tannic acid would have on the liver arose, knowing that acute poisoning leads to central acinar necrosis.

EXPERIMENTAL METHODS.

Twenty white rats aged about 3 months were used. Their average weight was 112 g., varying between 90 g. and 130 g. Male and female animals were used; they were all of the same strain, the origin of which was not known. The animals received a mixed diet of waste food from the hospitals, consisting mainly of milk-bread, potatoes, farinaceous food and sometimes fresh curd. No accurate diet could be maintained, so white rats, untreated or used for other experiments, were kept on the same diet and served as controls. Changes in the liver parenchyma could not be found either in the untreated rats or in those employed in other experiments, whether they died or were killed, though several animals were rather old at the time of the examination. The untreated animals grew and reproduced normally on this diet.

Treatment.

All rats were given 10 mg. of tannic acid on days 1, 3, 5 and 7 of the experiment. On days 9, 10, 15, 17, 19, 29, 51 and 53 the dose given was 20 mg., on days 60, 61, 93, 102, 109, 114 and 116 the dose was 30 mg., on days 65, 69, 84, 118, 123, 126, 132, 137 and 142 the dose was 40 mg., on days 147, 153, 159, 165, 172 and 194 the dose was 50 mg., on days 200 and 208 the dose was 60 mg., and on day 215, 70 mg. were given. The tannic acid was administered subcutaneously as a 1 per cent solution for the first 10 doses, and thereafter as a 2 per cent solution. Four animals died on day 19, 2 on day 29, 2 on day 31, 1 on day 34, 2 on day 39, and 1 on each of days 70, 71, 85, 94, 217 and 232. On each of days 69, 141 and 195 a rat was killed. The average weight fell from 112 g. to 96 g. in 19 days. On day 39 the average weight was 120 g., on day 69 it was 152 g., on day 102 it was 175 g., on day 137 it was 187 g., on day 166 it was 176 g., on day 190 it was 180 g., and on day 210 it was 195 g.

Necrosis occurred at the site of the subcutaneous injections after the 20th day, and ulcers 2 to 3 cm. diameter were left after separation of the necrotic tissue. All the ulcers healed without special treatment, and no inflammation of their margins or in the deep layers was observed. In no case could the death of the animal be attributed to these local processes.

All the animals were dissected as soon as possible. The organs of 18 rats were examined; 2 rats which died on days 19 and 39 had been devoured by the others.

Gross and microscopic changes in the liver.

In order to obtain a clear picture of the changes due to the prolonged treatment the animals were grouped according to the time of their death. The first group contained the animals which died up to the 39th day; the second group contained those which died between days 69 and 94; the animals in the third group survived 100 days.

Group I (9 rats).—The liver was slightly swollen, with a smooth surface, and had the structure of nutmeg liver in miniature. Histological examination showed a varying degree of central breakdown of the lobules. The débris had for the most part been removed and its place taken by red cells, resulting from dilatation or rupture of the central vein and neighbouring sinusoids. In the livers of those animals that died between 20 and 26 hours after the last treatment, karyorrhexis of the central cells could be seen. In the peripheral zone of the lobules the normal radial structure had disappeared. The liver cells were generally enlarged, nevertheless they displayed striking variations in size, and there were numerous cells in which both the cytoplasm and the nucleus were enormously enlarged (Fig. 1). In addition, double nuclei and cell divisions could be seen. The nuclear staining was uneven ; liver cells with pale vesicular nuclei were interspersed with others whose nuclei were hyperchromatic and pyknotic. Not infrequently round acidophil inclusion bodies could be seen in the vesicular nuclei. Large vacuoles appeared in the cytoplasm of some cells, but fat in small droplets could rarely be demonstrated in the sections with Sudan III. In some cases where there had been central haemorrhagic necrosis, accumulations of haemosiderin were present in the parenchymal cells. In other cases the cells contained light yellow granular bile pigment.

There were other changes deserving attention. As early as the 19th day there were in the sections small or medium-sized elongated cells with a bright staining nucleus, and these cells were more numerous in the livers of the animals which died later. These cells were arranged in short or long columns and strands between the remaining cords of liver cells. Sometimes they were situated on one side of the dilated central vein like a crescent or cap and they seemed to invade the liver tissue from here. As a rule, the number of these cells was increased in the periportal tissue also.

The behaviour of the reticulum as shown by Gömöri's impregnation was rather characteristic. Initially, the fibrous meshwork was collapsed in the region of the central acinar necrosis and broken to pieces on the periphery (Fig. 4). Later on, after the 30th day, the primary thickening of the reticulum fibres could be seen, mainly around the central veins, and to a lesser degree around the interlobular vessels (Fig. 5).

Group 2.—In 2 of the 5 rats belonging to this group (they died on days 71 and 85 respectively) the gross changes were well marked. The liver was enlarged

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EXPLANATION OF PLATES.

FIG. 1. Rat T/11. Treated with 180 mg. tannic acid administered in 11 doses. Died on 29th day. Haematoxylin and eosin. × 210.

FIG. 2.—Rat T/6. 18 injections, total 400 mg. tannic acid. Died on 85th day. Haematoxylin and eosin. × 360.

FIG. 3.—Rat T/18. 28 injections, total 750 mg. tannic acid. Killed on 141st day. Haematoxylin and eosin. × 54.

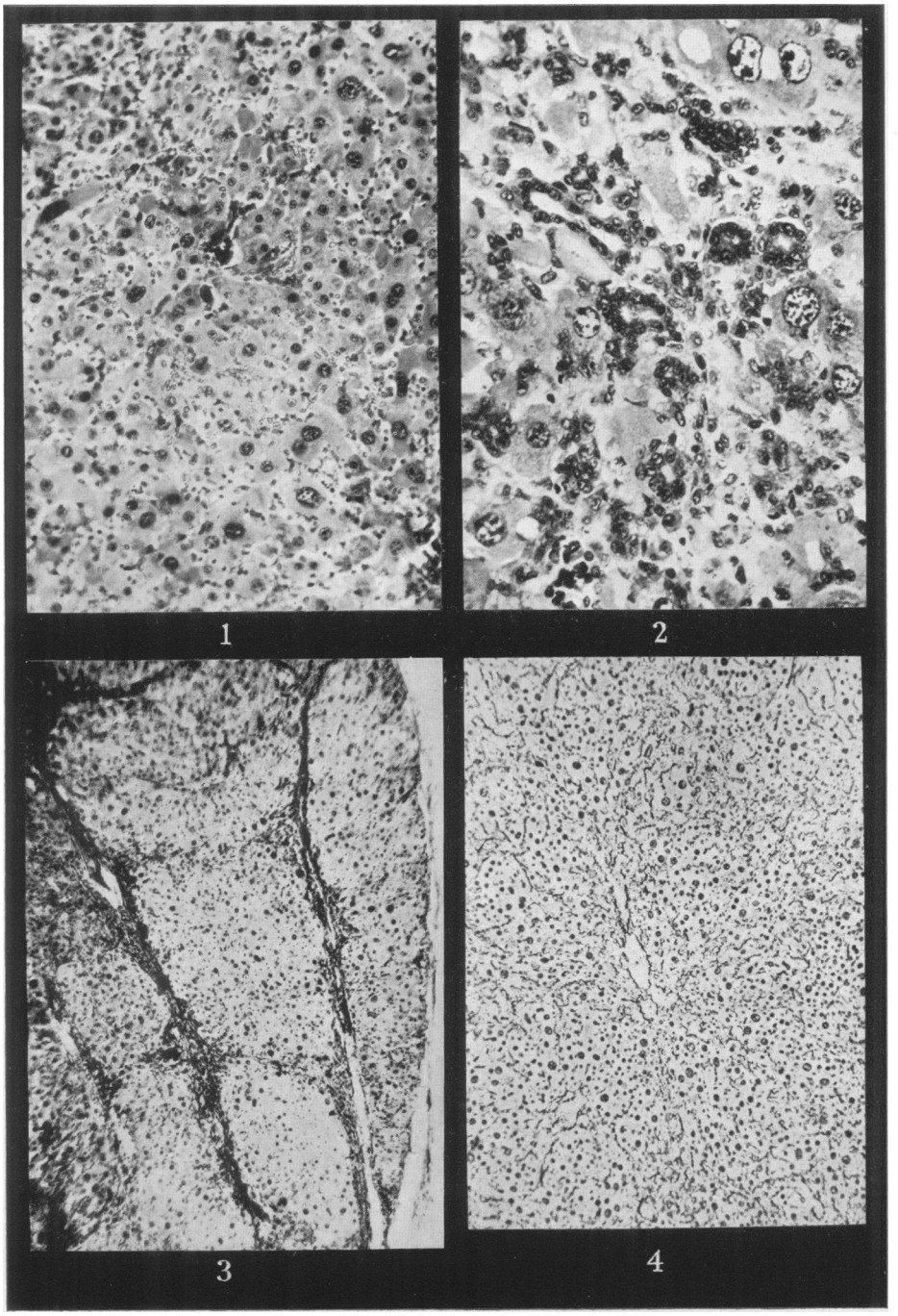
FIG. 4.—Rat T/11. 10 injections, total 160 mg. tannic acid. Died on 19th day. Gömöri's reticulum stain. × 57.

FIG. 5.—Rat T/13. Treated with 180 mg. tannic acid administered in 11 doses. Died on 31st day. Gömöri's reticulum stain. × 57.

FIG. 6.—Rat T/5. 16 injections, total 320 mg. tannic acid. Died on 69th day. Gömöri's reticulum stain. × 57.

FIG. 7.—Rat T/18. 28 injections, total 750 mg. tannic acid. Killed on 141st day. Gömöri's impregnation. × 57.

FIG. 8.—Rat T/19. 38 injections, total 1280 mg. tannic acid. Died on 217th day.



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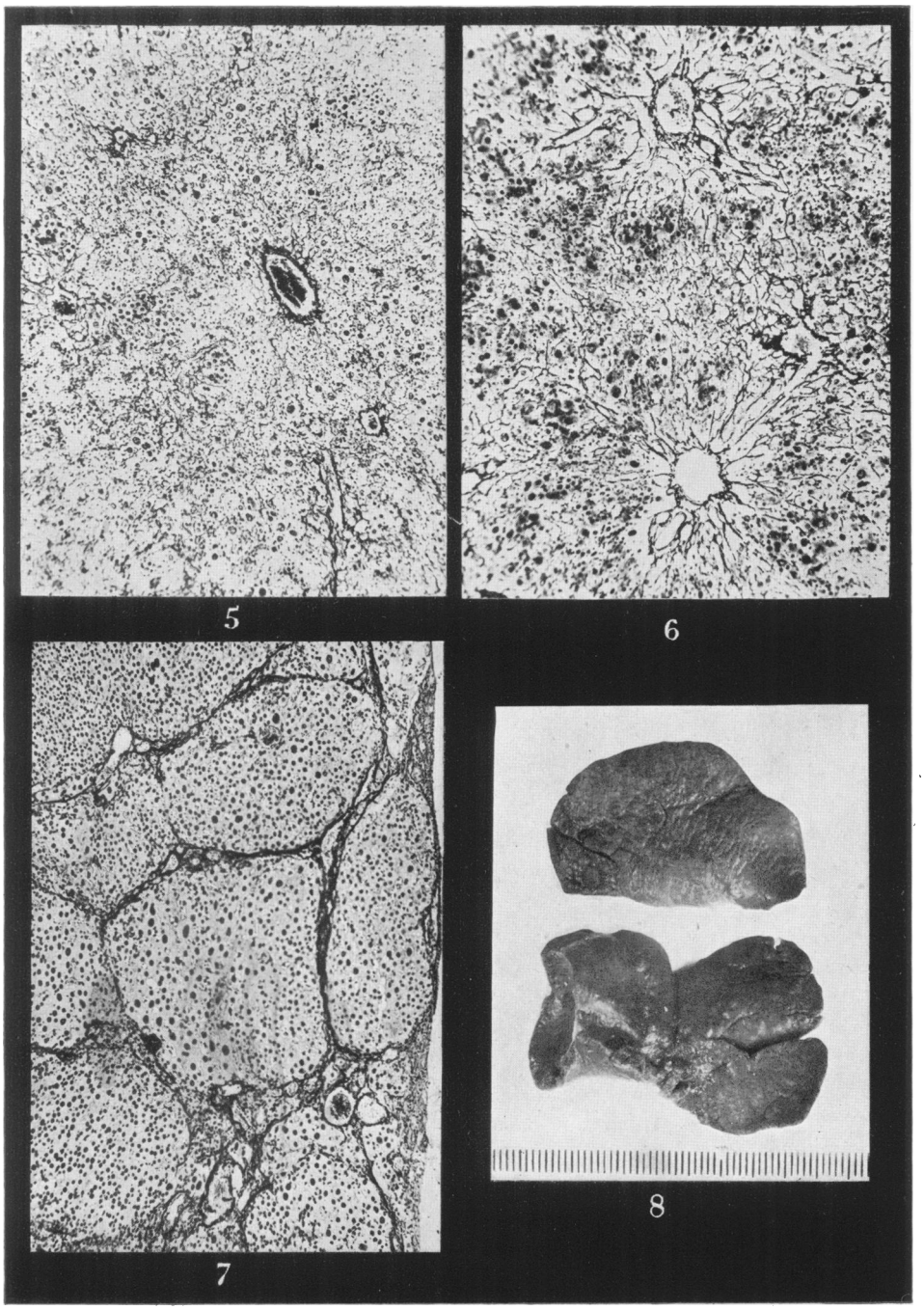

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and firm with its surface finely and evenly granular, especially when seen with a lens. The breakdown of the architecture of the liver was conspicuous in all cases and the polymorphism of the liver cells was more marked than in Group 1. The elongated cells mentioned under Group I were present in enormous numbers and not infrequently they formed thick strands extending from the centre of one acinus into another. In other places these cells were oval or flattened and here and there formed wreath-like structures.

The bile ducts were invariably increased in numbers, and groups of 8 or 10 were sometimes found between the lobules or in the interior of the disintegrated lobules. These bile ducts, some of which had no apparent lumen (Fig. 2), were often surrounded by the elongated cells previously mentioned. The reticulum fibres were likewise increased in numbers and thickened. The gradual transformation of the architecture of the liver could be followed well in sections stained by Gömöri's impregnation, a method which demonstrates the liver cells in addition to the reticulum. The thickened and more numerous reticulum fibres, forming strands of varying thickness, delineated and clearly indicated the irregular disintegrated lobules, and here and there surrounded groups of surviving liver cells (Fig. 6).

Sections of the liver of the rat which died on the 85th day, stained by van Gieson's method, showed collagen fibres in some of the thickened strands of connective tissue. Traces of fat and glycogen could also be found.

Group 3 (4 animals).—In 3 animals (treated for 141, 195 and 217 days respectively) it was obvious on external examination of the liver that it was diseased. The surface was finely and evenly granular, the greyish-brown nodules about the size of millet seeds were sharply defined and were separated by dark coloured furrows (Fig. 8). The cut surface showed that the granularity extended uniformly throughout the organ.

The histological picture was very similar to that of human cirrhosis of the Laennec type. The nodules corresponded to groups of liver cells surrounded by bands of connective tissue of varying thickness. These nodules are pseudo-lobules; the hepatic cells are not arranged in any definite fashion, and there is no central vein or the vein of the lobule is situated at the periphery (Fig. 3). The cells constituting these pseudo-lobules were markedly polymorphic, but the endothelium lining the rather wide sinusoids appeared normal. The pseudo-lobules had a delicate reticulum meshwork, in sharp contrast to the coarser surrounding fibres (Fig. 7). The small elongated cells were increased in number only in the environment of the liver cell groups. In two cases the proliferation of the bile ducts resulted in structures resembling adenomas.

In the liver of the rat which survived all the others, moderate fibrosis was present, but the characteristic distortion of the architecture was absent.

RESULTS.

Histological examination has shown that the protracted *parenteral* administration of tannic acid results in a gradually progressive destruction of the liver parenchyma and transformation of its architecture. In the early stages breakdown and subsequent regeneration of the liver cells are the most striking features; direct and indirect cell divisions may be seen in the marginal cells of the lobules, while other nuclei are, owing to their inner division, enormously enlarged. Changes

in the reticulum seem to be a good measure of the degree of transformation of the liver architecture. During the first week or two the reticulum fibres are moderately thickened and increased in numbers, particularly around the central veins. By the 70th day the increase and thickening of the reticulum is considerable and the broken down liver parenchyma is interwoven by strands of reticulum of varying thickness which split off pieces of varying size and shape from the lobules. At the same time the proliferation of the bile ducts sets in. This stage corresponds, in our opinion, to an early cirrhosis or precirrhosis.

In rats which survived 100 days of treatment the architecture of the liver was already distorted or completely transformed. The remnants of the parenchyma form round nodules of various sizes surrounded by a connective tissue meshwork containing collagen. The proliferation of the bile ducts simulates adenoma at many places. The changes seen in the liver of the rat killed on day 141 correspond to a diffuse fibrosis indistinguishable from the classical pattern of portal (Laennec) cirrhosis.

We attribute a particular role in this transformation of the liver structure (tannic acid cirrhosis) to the small elongated cells which were observed initially in small, later in gradually increasing, numbers. These cells are, in our opinion, of reticulo-endothelial origin and they seem to play some part other than reticulum formation. Our serial sections seem to indicate that these small immature cells may develop into bile ducts (i.e. epithelial cells of the bile ducts) and also occasionally those small liver cells which could be found in their environment. The presumption may be warranted that this strong hepatotoxic compound, or perhaps the repeated destruction of the parenchyma, has the sequel that the remaining reticulo-endothelium takes on the pluripotential properties of the embryonal mesenchyma.

DISCUSSION.

There are numerous papers in the literature dealing with the experimental production of cirrhosis. Besides the purely dietetic procedures mentioned in the introduction to this paper, there are many organic and inorganic substances—drugs, tar-like materials, proteins and their decomposition products, bacterial toxins, etc.—which produce liver lesions when administered to experimental animals (Moon, 1934). The results of these experiments are not quoted here as the lesions reported are quite unlike the classical human cirrhosis of Laennec type.

Mallory (1925, 1933; Mallory and Parker, 1931) was engaged in the problem of the aetiology of cirrhosis of the liver for several decades. Having ruled out ethyl alcohol as exclusively responsible, as other authors such as Friedenwald (1905) had also done, he performed extensive experiments to examine the effects of those substances which might contaminate alcoholic beverages. He examined more than 30 substances, including copper, lead, aluminium, antimony, zinc, arsenic, sulphates, furfurol, turpentine, and creosote. Ultimately, on the basis of his experiments in 1933 with phosphorus, he claimed that Laennec's cirrhosis was due to the contamination of beverages with phosphorus. However, the experimental phosphorus cirrhosis produced by Mallory bears little relation to human cirrhosis of the Laennec type.

There are, in fact, not many chemical substances capable of producing cirrhosis in a strict sense in animals. Beattie and Dickson (1948) hold that manganese

and its salts belong to the small group of substances which produce a hepatic lesion resembling human cirrhosis (Findlay, 1924; Hurst and Hurst, 1928). Many authors have of recent years been concerned with the effect of carbon tetrachloride (Cameron and Karunaratne, 1936; Sundareson, 1942; Ungar, 1945; and others). Ashburn, Endicott, Daft and Lillie (1947), unlike the other authors, contend that the first alteration due to carbon tetrachloride is proliferation of the connective tissue around the central veins. Since the discovery of Kinoshita (1937) attention has been directed to the cirrhogenic and carcinogenic effect of the azo dyes, especially butter yellow. The combination of different agents has proved more effective than the application of the individual substances (Moon, 1934).

Having compared the histological changes resulting from the protracted subcutaneous application of tannic acid, butter yellow, and other substances it appeared to us that the cirrhoses produced by carbon tetrachloride and butter yellow bore the greatest resemblance to tannic acid cirrhosis. The cirrhoses due to tannic acid and carbon tetrachloride may have a similar histogenesis, for in both the transformation of the architecture of the liver seems to start from the centre of the lobules. Nevertheless, wherever the process which results in diffuse fibrosis may begin, the final pattern cannot be distinguished from that of classical portal cirrhosis, as has recently been stressed with regard to dietetic cirrhosis (Glynn, Himsworth and Lindan, 1948).

Finally, the question arises whether the various substances which can produce cirrhosis in experimental animals play any part in the genesis of human cirrhosis. The majority probably play no part, but no definite opinion as to the aetiological role of tannic acid can be formed for the time being. Further investigations are being carried out in order to answer, amongst others, the following questions: (1) Have the skin necroses and ulcerations, which inevitably occur with the subcutaneous applications of tannic acid, any influence on the development of the cirrhosis? (2) Is the effect of oral administration identical with that of injection? In any case, we feel that the recognition of the fact that tannic acid can produce cirrhosis in experimental animals opens up a further line of investigation into this disputed problem.

SUMMARY.

1. The authors have produced diffuse nodular fibrosis of the liver in rats by the prolonged subcutaneous administration of tannic acid at various intervals.

2. As a result of the repeated administration of tannic acid, a gradual breakdown of the parenchyma and a progressive transformation of the liver architecture takes place. The first sign of this process is an increase of the reticulum around the central veins, corresponding with the central acinar destruction of the parenchyma.

3. By the 70th day of treatment fibre strands connecting adjacent lobules have been developed, and at the same time the regenerative proliferation of the bile ducts becomes conspicuous (precirrhosis).

4. The picture observed on the 141st day was indistinguishable from classical portal cirrhosis (Laennec type).

5. All rats dying between days 69 and 94 of the treatment developed changes corresponding to precirrhosis, whereas in 3 of the animals surviving 100 days a diffuse nodular cirrhosis was apparent on gross examination.

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THE KAOLIN-ADSORPTION METHOD FOR THE QUANTITATIVE ASSAY OF URINARY GONADOTROPHINS.

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THE concentration of gonadotrophic substances in normal male and female urine is so low, that for their assay concentrated extracts must be prepared, freed from toxic substances and other hormones, especially oestrogens and androgens. It has been found that the gonadotrophic substances of menopausal or castrate urine, and probably of normal urine as well, are more susceptible to any drastic extraction process than those of pregnancy urine. For example, 60 per cent acetone containing 4 per cent ammonia destroys the menopausal hormone but not the pregnancy hormone (Evans and Simpson, 1934). Therefore, mild methods of extraction must be used.

Crude extracts may be prepared by the original alcohol-precipitation method of Zondek (1930) as modified by Leonard and Smith (1934), Frank, Salmon and Friedman (1934), Heller and Heller (1939), Heller and Chandler (1942), Frank and Berman (1939) and Frank (1939), Varney and Koch (1942) and many others, by the tannic precipitation method elaborated by Levin and Tyndale (1936, 1937) or by the method involving adsorption on benzoic acid originated by