XXII. THE NITROGENOUS EXTRACTIVES OF TUMOURS.

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During the course of an investigation of the proteins of tumours, the results of which have already been reported by the author [1916], the protein-free aqueous extracts of a large number of tumours were obtained. In view of the scanty data recorded in the literature dealing with the extractive substances found in tumours, it was decided to make an examination of this material. The preparation of the extracts was carried out along the usual lines. The fresh tumour tissues in a finely minced condition were extracted with boiling distilled water, a trace of acetic acid being added to complete the removal of coagulable proteins. To the filtered extracts basic lead acetate was added until no further precipitation occurred, the resulting precipitate being filtered off, well washed, and the excess of lead removed from the filtrate by sulphuretted hydrogen.

The filtrate from the lead sulphide, freed from H_2S by passage of a rapid current of air, was acidified with sulphuric acid to the extent of 5 %, and the basic substances precipitated by the addition of a slight excess of a 30 % solution of phosphotungstic acid. After standing overnight in the refrigerator room the precipitate was filtered off and well washed with 5 % sulphuric acid. Each such precipitate was dried in a desiccator over sulphuric acid and stored in this form until sufficient material of each class had been collected for examination.

The filtrates from the phosphotungstic acid precipitations were quantitatively freed from the excess of the precipitating reagent and sulphuric acid by means of baryta, filtered and evaporated to small bulk by distillation *in vacuo* at 40° . In this concentrated form the fractions were stored at 0° , until sufficient had been collected.

The tumours examined were a number of human tumours of mammary origin, a lymphosarcoma, a large secondary deposit in the liver from a pancreatic carcinoma and a series of the avian neoplasm known as the Rous chicken sarcoma.

I. EXTRACTIVE NITROGEN OF THE ROUS CHICKEN SARCOMA.

By the method just described it was found possible during the course of several months to collect fractions from the aqueous extracts of 4320 g. of this tumour.

The tumours were grown by the inoculation of grafts of the tumour into the pectoral muscle of healthy, adult, pure bred, fowls of the Plymouth Rock strain. The resulting tumours grow rapidly, infiltrating and destroying the adjacent muscle tissue, so that after some three weeks' growth the tumour has usually attained the size of a hen's egg and weighs 100 g. or more. Under conditions which result in a very rapid rate of growth, such as implantation into young birds, the tumours usually undergo extensive central degeneration during the latter stages, and the cavity of the growth becomes filled with a thick, mucoid, blood-stained fluid.

I (a). *Phosphotungstate Precipitate.*

The combined precipitates of phosphotungstates from the extracts of the 4320 g. of tumour tissue weighed in the dry condition 225 g. After being ground to a fine state of division and passed through a fine mesh sieve, it was decomposed in aqueous suspension by means of a hot concentrated solution of baryta. The clear filtrate was quantitatively freed from the excess of barium by sulphuric acid and evaporated down by distillation under reduced temperature and pressure to a thick syrup. The residue was submitted to extraction with hot alcohol, thus removing 5.2 g. of a dirty white substance, insoluble in that solvent and mainly inorganic in nature. The alcohol was removed from the combined extracts by distillation and the residue taken up in a litre of distilled water. This solution was then fractionated by the use of silver nitrate and baryta into four fractions:

- I (a) i. Purine fraction.
- I (a) ii. Histidine fraction.
- I (a) iii. Arginine fraction.
- I (a) iv. Lysine fraction.

The last fraction consisted of the re-precipitated phosphotungstates after the separation of the first three fractions and the subsequent removal of the excess of silver and baryta.

I (a) i. *Purine Fraction.*

The purine silver compounds were decomposed in aqueous suspension by means of sulphuretted hydrogen gas and the silver-free filtrate concentrated down to dryness at reduced temperature and pressure.

The resulting dirty white residue was dissolved in a small volume of dilute hydrochloric acid, filtered and rendered alkaline with ammonia. On standing, a very small quantity of a dirty granular product separated out. Insufficient was obtained to permit of satisfactory purification and analysis, but its reactions, and the isolation of the crystals of the hydrochloride and phosphotungstate, were sufficient to identify the substance as guanine. After neutralisation of the filtrate and the addition of picric acid solution, a crop of crystals of adenine picrate was obtained. Recrystallised from water, 0.7 g. of the purified picrate was isolated (M.P. 273°). Analysis by means of the nitron method for the estimation of picric acid gave the figures:

0.0264 g. adenine picrate; 0.0372 g. nitron picrate; 59.5 % picric acid.

Calculated for $C_5H_5N_5$, $C_6H_3O_7N_3$, H_2O ... 60.0 % „ „

The filtrate from the adenine picrate, freed from the excess of picric acid by acidification with nitric acid and extraction with toluene, was evaporated almost to dryness, when typical crystals of hypoxanthine nitrate separated out. After recrystallisation from dilute nitric acid, 0.4 g. of the pure salt was obtained. No xanthine or other substance was isolated from this fraction.

I (a) ii. *Histidine Fraction.*

The silver compounds were decomposed in aqueous suspension by H_2S gas, and the neutralised, silver-free filtrate evaporated to dryness at reduced temperature and pressure. The small residue was extracted with hot alcohol, thus removing a small amount of insoluble inorganic salts, and the alcoholic extracts were freed from alcohol under reduced pressure. The residue gave positive reactions for histidine. Accordingly it was dissolved in 100 cc. of distilled water, and mercuric sulphate solution added until no further precipitation occurred. The small mercury precipitate was filtered off and decomposed in aqueous suspension by H_2S gas, the filtrate quantitatively freed from sulphuric acid and evaporated down on the water-bath with dilute hydrochloric acid. After standing for several days in the desiccator, a very small yield of the typical crystals of histidine hydrochloride was obtained, which, after one recrystallisation from dilute alcohol, melted at 251°. From the hydrochloride the picrolonate was prepared, twice recrystallised from hot

water, and analysed by the micro-Dumas method of Pregl, after being dried *in vacuo* at 110° for 2 hours (M.P. 224°).

3.57 mg.; 0.695 cc. N gas (19°, 766 mm.) 23.2 % N

Calculated for $C_6H_9O_2N_3$, $C_{10}H_8O_5N_4$... 23.45 % N

Although there was evidence of the presence of other substances in the histidine mother liquors, attempts to isolate these in a pure condition failed.

I (a) iii. *Arginine Fraction.*

The silver salts were decomposed in the usual manner in aqueous suspension with H_2S gas, and the silver-free filtrate evaporated down to small bulk at reduced temperature and pressure. No crystalline product could be isolated direct, but after the addition of a solution of picrolonic acid and on standing some days, a small amount of a micro-crystalline picrolonate was obtained. After recrystallisation from hot water 0.12 g. of the salt was yielded, which showed a melting point of 255° and gave, on analysis, figures which indicated that it was probably an impure preparation of arginine picrolonate. Attempts to purify the salt and analyse the purer product were unsuccessful.

The filtrate from this picrolonate was freed from the excess of picrolonic acid by extraction of the acidified solution with ether and concentrated down to a small bulk. On the addition of a saturated aqueous solution of picric acid a very small crop of a pale yellow crystalline picrate was deposited. After one recrystallisation from water the picrate melted at 214°, and was identified as creatinine picrate by the reactions of the hydrochloride prepared from the picrate. The occurrence of creatinine in this fraction is probably due to its being carried down during the precipitation of the silver compounds.

I (a) iv. *Lysine Fraction.*

The reprecipitated phosphotungstates comprising this fraction were decomposed in the usual manner by means of baryta, the excess of barium quantitatively removed, and the filtrate concentrated *in vacuo* at reduced temperature. Hot alcohol extraction of the resulting syrup left behind a considerable amount of insoluble inorganic salts. The hot alcohol extracts on cooling deposited a crop of a pure white crystalline substance, which was filtered off and recrystallised from hot alcohol. The purified substance separated out in clusters of needles and showed a melting point of 244°. Its reactions corresponded with those of carnosine, and its identity with that substance was established by the isolation of the characteristic copper salt, and the analysis of the free base.

0.0589 g. (dried 110° *in vacuo*); 10.20 cc. 0.1 *N* NH_3 ... 24.2 % N
 Calculated for $\text{C}_9\text{H}_{14}\text{O}_3\text{N}_4$... 24.6 % N

After separation of the carnosine, it was found that the alcoholic filtrate gave a copious crystalline precipitate with alcoholic picric acid. The picrate after standing overnight at 0° , was filtered off and recrystallised from water, 16 g. of the pure compound being thus obtained. The product was readily identified as creatinine picrate, M.P. 213° .

0.0765 mg. of the picrate, dried at 110° for 2 hours *in vacuo*;

0.1205 mg. of nitron picrate ... 66.7 % picric acid.

Calculated for $\text{C}_4\text{H}_7\text{ON}_3$, $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$... 67.0 % „ „

The hydrochloride prepared from the picric acid salt melted at 264° , and gave all the reactions for creatinine hydrochloride.

No other substance was isolated from this fraction except a small yield of what was probably impure leucine.

I (b). *Phosphotungstic Acid Filtrate.*

The concentrated fractions, prepared as already described, had been stored in the refrigerator room and upon examination it was found that a considerable crystalline deposit had separated out. This consisted mainly of tyrosine, but typical crystals of leucine were also identified as being present. After recrystallisation 0.6 g. of fairly pure tyrosine was obtained and recognised by its reactions.

The filtrate from the tyrosine was concentrated to a thick syrup under reduced temperature and pressure, another small yield of tyrosine being obtained during the concentration, and taken up in hot alcohol.

The alcoholic extracts gave a heavy crystalline precipitate on the addition of alcoholic picric acid. The deep yellow picrate, recrystallised from water, showed an imperfect melting point at $236\text{--}250^{\circ}$, and was identified as creatinine potassium picrate.

0.0970 g.; 0.1731 g. of nitron picrate ... 75.5 % picric acid.

0.0953 g.; 0.1702 g. „ „ ... 75.6 % „ „

Calculated for $\text{C}_4\text{H}_7\text{ON}_3$, K, $(\text{C}_6\text{H}_3\text{O}_7\text{N}_3)_2$ 75.3 % „ „

Creatinine hydrochloride, with a melting point of 258° , was prepared from the double picrate.

Attempts to separate other substances in a pure condition from this fraction failed, although several impure products were isolated, of which one was apparently an impure specimen of leucine, whilst another was probably a still more impure preparation of glycine.

II. EXTRACTIVE NITROGEN OF MAMMARY CARCINOMATA.

The chief difficulty encountered in this investigation was the collection of sufficient material. Altogether, some 600 g. of mammary tumour tissue were available, but it was not all of the same type, as was the large amount of chicken tumour used in the first section of the work. The majority of the specimens—weighing together about 450 g.—were primary carcinomata of the mammary gland. Of these 75 % were of the slow growing scirrhus type, the remainder being more or less encephaloid in character. The remaining 150 g. of tissue examined was made up of carcinomatous deposits in the axillary glands, secondary to primary growths in the breasts, and one myxosarcoma of the same organ. The combined aqueous extracts of these tissues were treated by the process described in the first portion of this paper.

II (a) *Phosphotungstate Precipitate.*

The combined phosphotungstate precipitates weighed only 35 g. They were reduced to a fine state of division and decomposed in aqueous suspension by means of baryta. The neutralised filtrate was then sub-fractionated, as described in the first section of this paper (p. 247), into four fractions.

Owing to the fractions being so very small, the separation of substances in a pure condition from them was rendered exceedingly difficult.

From the purine fraction II (a) i, after an attempt at purification of the substances present by the copper bisulphite process, a very small yield of adenine picrate was obtained, M.P. 270°, but no other substance was isolated.

The second and third fractions, II (a), ii and iii, although worked up as carefully as possible, yielded no pure products. A substance giving a strong coloration with diazo-benzenesulphonic acid, and precipitated by mercuric sulphate, was detected in the histidine fraction; whilst from the arginine fraction a small yield of a very impure picrolonate was obtained, but in both cases the amount was insufficient to permit of identification.

From the lysine fraction, treated in a similar manner to the corresponding fraction from the Rous extract, a small yield of creatinine picrate was isolated. After purification 0.2 g. of the salt was obtained, M.P. 216°, from which a hydrochloride was prepared, showing the typical reactions and a M.P. of 261°. From the creatinine mother liquors a very small yield of a picrate melting at 180—190° was isolated, but the quantity was too small to permit of purification or identification.

II (b). *Phosphotungstic Acid Filtrates.*

From the phosphotungstic acid filtrates, decomposed and stored as already described, a small yield of tyrosine had separated out. This was removed, the filtrate evaporated to dryness and the residue taken up in alcohol. On the addition of alcoholic picric acid, and standing, a very small yield of creatinine picrate in a fairly pure condition was obtained. This was identified by its melting point, 214° , and by the reactions of the free base prepared from the picric acid salt. No other substance was isolated in a pure condition from this fraction.

III. EXTRACTIVE NITROGEN OF A LYMPHOSARCOMA.

This tumour, weighing some 900 g., was an exceedingly rapidly growing sarcoma of the cervical glands occurring in a young man. The growth was firm and showed little evidence of necrotic change having occurred. The aqueous extract of 800 g. of the tumour was examined.

III (a). *Phosphotungstic Acid Precipitate.*

This precipitate weighed 29 g. in the dry state, and was decomposed in the usual manner. The resulting solution of bases was fractionated by the silver method.

III (a) i. *Purine Fraction.*

This was the largest silver fraction. It was decomposed and the purine bases reprecipitated by the copper bisulphite process. From the solution of the purine hydrochlorides, obtained by decomposition of the copper compounds, ammonia precipitated 0.02 g. guanine. This was identified by its reactions and the crystalline form of its hydrochloride. From the guanine filtrate picric acid precipitated a fairly heavy yield of adenine picrate. After one recrystallisation, 0.42 g. of the picrate, melting at 272° , was obtained. From the oxy-purine fraction a very small yield of impure xanthine was separated, but no evidence of the presence of hypoxanthine was obtained.

III (a) ii and iii. *Histidine and Arginine Fractions.*

These fractions were very small and no pure substance was isolated from them.

III (a) iv. *Lysine Fraction.*

This fraction was worked up in a similar manner to other lysine fractions, but only a small yield of creatinine in the form of its picrate (0.2 g., m.p. 214°) and traces of tyrosine and leucine were isolated.

III (b). *Phosphotungstic Acid Filtrate.*

From this fraction 0.12 g. tyrosine and 0.65 g. leucine were separated in a fairly pure condition.

IV. EXTRACTIVE NITROGEN OF CARCINOMA OF PANCREAS.

The material for this examination was a mass of secondary deposits in the liver from a primary carcinoma of the pancreas. The primary growth weighed but 29 g., whilst nearly 5 kilos. of tumour tissue were obtained from the liver deposits. These growths had undergone somewhat extensive central necrosis, with the formation of caseous material. The aqueous protein-free extract of 3.5 kilos. of this material, excluding as far as possible the more extensively degenerated areas, was examined.

IV (a) i. *Purine Fraction.*

This fraction was fairly large and was submitted to an initial purification by the copper bisulphite process. Guanine was isolated in very small amount, 0.07 g. of the somewhat impure base being precipitated by ammonia. This was identified in the usual manner. From the guanine filtrate picric acid threw down a small precipitate of adenine picrate. After recrystallisation 0.25 g. of the purified salt was obtained (M.P. 275°).

After removal of the picric acid from the adenine filtrate the oxy-purines were reprecipitated by the copper bisulphite procedure.

From the decomposed copper compounds 0.03 g. of xanthine and 0.06 g. of hypoxanthine nitrate were isolated by the usual methods.

IV (a) ii and iii. *Histidine and Arginine Fraction.*

These fractions were very small in quantity and no substances were isolated in a pure condition from them. Indications of the presence of histidine were observed, but the amount present was too small to permit of its isolation or identification.

IV (a) iv. *Lysine Fraction.*

The reprecipitated phosphotungstates constituting this fraction weighed in the dry state 27 g. The solution of free bases obtained by decomposition of this precipitate gave upon concentration and treatment with picric acid solution a small yield of creatinine picrate. After one recrystallisation 0.37 g. of the pure picrate was obtained (M.P. 217°).

After freeing the solution from picric acid and further concentrating, a small yield (0.07 g.) of tyrosine was isolated in a fairly pure condition. Traces

of impure leucine were also separated from the residue, which, however, was largely inorganic in nature.

IV (b). *Phosphotungstic Acid Filtrate.*

Leucine (0.44 g.) and tyrosine (0.27 g.) were isolated from the concentrated filtrate of this fraction. From the mother liquors picric acid precipitated a further small yield of creatinine picrate (0.11 g. purified salt, m.p. 214°).

No other substance was isolated from this extract.

DISCUSSION OF RESULTS.

The results of the four examinations recorded above agree with the scanty published data on tumour extractives. Saiki [1909] examined a mixed batch of tumours, consisting of human carcinomata from various sources, and weighing in all some 200 g. and found 0.001 % of purine nitrogen in the protein-free aqueous extracts. He was unable to isolate the individual purines owing to the small amount of material at his disposal. In another trial he found that the purine bases present in 570 g. of mixed cancerous tissue, mainly carcinomata of the ovary, were as follows: 0.1391 g. uric acid, 0.1866 g. adenine, 0.0190 g. hypoxanthine, and traces of guanine and xanthine. He suggests that these observations indicate the presence of guanase and xanthoxidase, and the absence of adenase in human tumours.

Wells and Long [1913] found that the purine content and purine enzymes of human tumours, both malignant and benign, resembled those of normal tissues, and that xanthoxidase was absent. They found it difficult to explain the high percentage of uric acid found by Saiki, since the latter author gives practically no details of the methods employed in the isolation or identification.

Saiki also records finding creatinine to the extent of 0.016 % in fresh cancer tissue, and very small amounts of creatine.

The presence of considerable amounts of carnosine and creatinine in the extract of the Rous chicken sarcoma may be attributed to some extent to the extensive degradation of muscle tissue consequent on the tumour invasion.

Autolysis is probably responsible for the presence of the amino-acids, for Beebe [1904] found leucine, tyrosine, glycine and tryptophan in tumour extracts.

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