CCLXIX. THE DISTRIBUTION OF CHOLINE.

By JOHN PALMER FLETCHER, CHARLES HERBERT BEST
AND OMOND McKILLOP SOLANDT.

From the Department of Physiological Hygiene, School of Hygiene, University of Toronto.

(Received August 1st, 1935.)

INTEREST in the total choline content of animal and plant tissues has been aroused by recent investigations which have demonstrated the importance of choline as a dietary constituent. Previous estimates of the choline content of various tissues have been largely concerned with the determination of free choline [Alles, 1934; Guggenheim, 1924]. More recently there have been some attempts to measure free and bound choline separately. The methods used for the separation of free and bound choline are complex and tedious and the separation is apparently unnecessary for the purpose of dietetic experiments on normal animals. Consequently an attempt has been made to evolve a reasonably accurate and rapid method for the estimation of the total choline content of foods. The present paper is concerned with the details of the method devised and some of the results obtained with it. In addition to measuring the total choline content of many materials which have been incorporated in experimental diets for rats and dogs, the total choline content of the different tissues of the normal white rat was investigated.

Method.

After careful consideration of all the chemical and biological methods for the estimation of choline it was decided that extraction, acetylation and assay of the resultant acetylcholine, using the isolated intestine of the rabbit, was the most advantageous method for the present purpose. A description of the method finally evolved may be divided into two parts. First, the digestion of the tissue, hydrolysis of choline-containing compounds and extraction and acetylation of the choline; second, the biological assay of the resulting acetylcholine.

Digestion and acetylation.

The hydrochloric acid digestion used by Best and McHenry [1930] for the estimation of histamine in tissues has proved to be equally satisfactory for the determination of choline. This procedure breaks up the tissue, hydrolyses the choline-containing compounds and extracts the choline without destroying measurable amounts.

The technique finally adopted is as follows. 2–4 g. or more of fresh tissue or other material are rapidly weighed, minced and transferred to a 1-litre, round-bottomed flask; 20–40 ml. of 18 % HCl are then added and the mixture is boiled under a reflux condenser for 1 hour. After the boiling, 30–60 ml. of 95 % ethyl alcohol are added and the mixture of water, alcohol, acid and ester is removed *in vacuo* at about 90°. The residue is then washed with a small amount of 95 % alcohol and finally with absolute alcohol. The alcohol is removed *in vacuo* as before.

The residue, which has been carefully dried after the final washing with absolute alcohol, is then acetylated by the method of Abderhalden and Paffrath [1925]. 25 ml. glacial acetic acid and 5 ml. acetic anhydride are added to the residue and the mixture is boiled under a reflux condenser for 2 hours. The acetic acid and anhydride are then removed *in vacuo* at about 90° and the residue washed once with absolute alcohol. The acetylated residue is finally transferred to a volumetric flask by alternate small washings of alcohol and water until the total volume is 100 ml. This alcoholic solution is then diluted as required for assay.

Each step in this procedure has been tested by control experiments. The results of these experiments may be summarised as follows.

1. Many experiments on the destruction of choline during tissue autolysis suggest that no special precautions are necessary to prevent loss of choline between the time of death of the animal and the beginning of digestion of the tissue.
2. Varying the time of acid digestion from 30 to 90 min. caused no change in the value for the choline content of pancreas, suggesting that hydrolysis and extraction are complete in the shorter time.
3. Pure choline chloride is not measurably affected by 90 min. boiling with 18 % HCl.
4. The method of acetylation repeatedly gave perfect acetylation of pure choline chloride solutions within the limits of accuracy of the biological assay.
5. Recovery of choline chloride added to tissues has always been complete within the limits of accuracy of the method.

The biological assay.

The isolated intestine of the rabbit was used as the test object for all assays. The intestine was mounted in a double intestine bath of conventional design and was bathed in Ringer-Tyrode's solution containing no glucose. It was found that better results were obtained with the duodenum than with any other part of the intestine. The intestine was definitely more reliable when removed under ether anaesthesia than when it was obtained from the dead animal. Purity of the Tyrode's solution and accuracy of temperature control are of course essential to satisfactory assaying.

All assays were done against a standard acetylcholine solution. The potency of this standard was frequently checked against fresh solutions and against freshly acetylated choline.

The accuracy of the biological assay was tested by assaying other dilutions of the standard against that ordinarily used. The results of these tests never showed an error greater than 10 % and there did not seem to be any significant constant error. To attain this accuracy it is essential that doses of acetylcholine be chosen which will cause a contraction which is about 75 % of the maximum. The concentration of acetylcholine necessary to elicit such a contraction varies considerably with different preparations but is usually one part of acetylcholine in 1 to 3×10^{-8} parts of solution.

In discussing the accuracy of the biological assay of the acetylated tissue residues, a great many interfering substances must be considered [Chang and Gaddum, 1933]. The prolonged heating and exposure to hydrochloric acid involved in the digestion process almost certainly destroy the adenosine derivatives [Drury and Szent-Györgyi, 1929], the substance P [Euler and Gaddum, 1931] and callicrein [Frey and Kraut, 1928; Kraut *et al.*, 1930]. Two samples

of creatinine were tested on the rabbit intestine. In doses up to 5000 times the usual dose of acetylcholine they caused no contraction at all.

The method of extraction used does not destroy histamine so that all the histamine from the tissues appears in the final solution. The rabbit intestine is very insensitive to histamine, but in order to be certain that the histamine would not have any significant effect on the results the histamine/acetylcholine ratio was determined by direct assay. Ratios were also obtained for choline and betaine. The results of these assays are given in Table I.

Table I. *Potency ratios determined with the isolated intestine of the rabbit.*

Substance	Ratio
Acetylcholine	1
Acetyl-β-methylcholine	1·7
Histamine	1,300 2,700 3,250
Choline	3,500 4,200 5,500 6,100
Betaine	70,000

The figures given are the dose of the material required to cause a contraction of the intestine of the same magnitude as that caused by a unit dose of acetylcholine.

Among other substances which might interfere are acetate ions and ethyl alcohol. In most of the assays performed the amount of acetylcholine present was so great that, in the solution actually used for assay, the concentration of these substances was entirely negligible. In all cases where the concentration of acetylcholine was low enough to suggest some danger of interference, the solutions were assayed both before and after the addition of atropine to the perfusing fluid. In addition to the assay after atropine, some of the solutions were assayed before acetylation.

This combination of acid digestion, acetylation and assay on the isolated intestine of the rabbit seems to result in a method for the estimation of the total choline content of tissues which is quite specific for choline. The accuracy of the results obtained is probably limited chiefly by the accuracy of the biological assay in all cases where the amount of choline in the substance being examined is moderately large. Where the amount of choline present is very small the presence of interfering substances may produce an appreciable error. The maximum error in the assay of the acetylated tissue residues is apparently about $\pm 15\%$ since duplicate assays have occasionally differed by almost 30%. With careful technique differences of this magnitude are very rarely encountered. In most of the present work duplicate assays were done on at least two samples of the material being analysed so that the average result should be correct within less than $\pm 10\%$.

Results.

The results which are reported here include the total choline content of the tissues of the normal white rat, of a variety of other animal tissues and of some materials used in experimental diets for rats and dogs. In addition, many other total choline determinations, done by the method which has been outlined, have been or will be reported in other communications. [Best *et al.*, 1934; Best, MacLean and Ridout, 1935; Best, Huntsman, McHenry and Ridout, 1935; McHenry, 1935, *etc.*]

The total choline content of rat tissues.

The results of the choline estimations on rat tissues are given in Table II. The rats used were all young adult white rats of the Wistar Institute strain. They had been reared at the Connaught Laboratories Farm on a commercial

Table II. *Total choline content of rat tissues.* (*Adult, white.*)

No.	Tissue	No. of exps.	Total no. of rats	Average choline content mg./100 g.
1	Spermatic fluid	2	5	514
2	Spinal cord	2	2	370
3	Brain	4	10	325
4	Adrenals	6	15	304
5	Cerebellum	2	5	296
6	Cerebral hemispheres	2	4	274
7	Liver	28	28	260
8	Pancreas	5	14	232
9	Pituitary	4	8	224
10	Kidneys	8	9	202
11	Thyroid	4	11	167
12	Lungs	3	5	164
13	Heart	4	8	158
14	Lymph glands	1	3	152
15	Stomach	3	4	152
16	Spleen	4	7	151
17	Small intestine	5	6	142
18	Large intestine	2	3	139
19	Salivary glands	1	2	131
20	Tongue	2	6	123
21	Thymus	2	3	113
22	Skeletal muscle	4	9	100
23	Uterus	3	5	74
24	Skin	2	2	64
25	Bone	2	4	44
26	Connective tissue	1	1	40
27	Fat	5	6	23
28	Blood—Starved	3	4	22
	Fed	1	1	31

No difference was detected between the choline contents of tissues from male and female animals.

"balanced ration". The average weight of the rats used was about 200 g. The rats were starved for 24 hours before use. In most cases corresponding tissues from several rats were pooled for the choline estimations.

The tissues are listed in the table in order of choline content. The results show that sperm-containing fluid from the seminal vesicles has the highest choline content of any tissue examined. This high choline content makes the values obtained for epididymis, ductus deferens and seminal vesicles of little significance since the result probably depends largely upon the content of seminal fluid.

The results obtained for the various parts of the central nervous system are quite in keeping with the high phospholipin content of these structures. It is interesting to note that the adrenal gland has approximately the same choline content as the other structures of nervous origin.

The average value given for the choline content of the liver is based on many more estimations than are the other results. In one experiment the livers of 24 rats were tested individually. These rats had been on an adequate mixed diet and were fully grown, averaging 250 g. in weight. The average choline content of the livers of these 24 rats was 260 mg. per 100 g. of fresh tissue.

The figure for the choline content of rat's pancreas may be too low since considerable difficulty was experienced in separating the pancreas from the connective tissue in which it is embedded.

The acetylated product from the lymph glands gave a very large, delayed contraction of the intestine after the contraction due to acetylcholine had subsided. This delayed contraction was not eliminated by atropine. Although the delayed contraction was not considered in calculating the result it is possible that the substance causing the contraction may have affected the result obtained.

The fat used in the choline estimations was all intra-abdominal fat and was obtained from around the kidneys, testicles or uterus and from the mesentery.

The total choline content of other animal tissues.

In Table III are given the total choline contents of a variety of tissues from animals other than the rat. Most of these results require no comment. The results for the choline content of dog stomach were obtained on material prepared for histamine assay [Gavin *et al.*, 1933]. This process involves neutralisation and filtration, which is not included in the ordinary technique for choline estimations, so the result may be slightly lower than would otherwise be the case.

Table III. *Total choline content of various animal tissues.*

Animal	Tissue	No. of samples	No. of determinations	Choline content mg./100 g.
Ox	Liver	1	2	270
	Pituitary—Anterior lobe	3	3	259
	Posterior lobe	3	3	217
	Pancreas	7	26	230
	Muscle	1	1	76
	Blood (defibrinated)	1	2	13
	Fat	2	3	0·5–2·6
Dog	Liver	4	19	230
	Stomach	2	4	90
	Blood (whole)	5	11	34
Pig	Pancreas	1	4	280
	Bacon (cured side bacon)	1	1	44
	Fat (from cooking bacon)	1	2	6
	Lard	1	2	1
Codfish	Muscle	2	2	78

The total choline content given for dog's blood is the average of 10 determinations on 5 samples of blood from 4 different dogs. The dogs were not starved before the blood was drawn. The results obtained ranged from 27 to 39 mg. of choline per 100 ml. of whole blood, with an average value of 34 mg. per 100 ml.

The total choline content of various foods.

The total choline content of a considerable number of foods has been investigated in a search for suitable ingredients for low-choline diets for rats and dogs. The results of the total choline estimations on some of these materials are given in Table IV. Those substances which are stated to contain no choline contain less than 0·1 mg. per 100 g. of material. The acetylated products from sugar and potato starch caused a smooth, rapid contraction of the intestine, not unlike that caused by acetylcholine, but this contraction was not diminished by atropine. Hence sugar and potato starch were considered to contain no significant amount of choline.

Table IV. *The total choline content of various cereals and other materials used in experimental diets.*

Material	Remarks	Choline content mg./100 g.
Flour	White wheat flour	140
Dog biscuit	Spratt's commercial grade	130
Oxo	Commercial meat extract	105
Rice	Polished	94
Milk powder	Dried, skimmed milk	90
Bovril	Commercial meat extract	78
Rice flour	Various commercial brands	73–65
Caseinogen	"Lister's prepared casein"	70
Bone meal	Commercial grade	30
Washed bran	—	28
Corn starch	—	25
Cheese	Canadian cheddar	19
Egg albumin	—	18
Rice starch	Various commercial brands	15–4·3
Butter	Fresh creamery butter	13
Caseinogen	British Drug Houses, "fat- and vitamin-free"	3·5
Egg white	White separated from hard-boiled eggs	2·0
Cellu flour	Ground cellulose	1
Edestin	Pfanstiehl, "pure"	1
Agar-agar	Various commercial brands	1·6–0·8
Crisco	Hydrogenated vegetable oils	0·4
Potato starch	Various commercial brands	0
Cane sugar	Various commercial brands	0
Mazola	Refined corn oil	0
Olive oil	—	0

Table V. *Total choline content of vitamin concentrates and vitamin-rich foods.*

Material	Remarks	Choline content mg./100 g.
Baker's yeast	Dried and powdered	270
Brewer's yeast	Dried and powdered	240
Radiomalt	—	64
Turnip	Fresh—used as source of vitamin C	42
Vitamin B_1 concentrate	Prepared according to method of Kinnersley and Peters (4 samples)	22–8
Cod-liver oil concentrate	Vitamin A—500,000, vitamin D—3,000 International Units per g.	14
Tomato juice	Various commercial brands	9·8–6·6
Vitamin E oil	Unsaponifiable matter of wheat germ	4·0
Vitamin B_1 concentrate	Fuller's earth adsorbate from an extract of rice polishings	1·2

In Table V are given the total choline contents of a variety of vitamin-rich foods and vitamin concentrates which were investigated in connection with the preparation of the diets low in choline.

Summary.

A method for the estimation of the total choline content of tissues is described. The method consists of digestion of the tissue with hydrochloric acid, acetylation of the extracted choline and assay of the resulting acetylcholine on the isolated intestine of the rabbit. The use of the hydrochloric acid digestion is the only novel part of the method. Acetylation and assay are carried out by well established procedures.

The total choline contents of the various tissues of the normal white rat, of several tissues from other animals and of many dietary constituents of both animal and vegetable origin have been determined by this method.

REFERENCES.

Alles (1934). *Physiol. Rev.* **14**, 278.
Abderhalden and Paffrath (1925). *Pflüger's Arch.* **207**, 228.
Best, Channon and Ridout (1934). *J. Physiol.* **81**, 409.
—— Huntsman, McHenry and Ridout (1935). *J. Physiol.* **84**, 38 *P.*
—— and McHenry (1930). *J. Physiol.* **70**, 349.
—— MacLean and Ridout (1935). *J. Physiol.* **83**, 275.
Chang and Gaddum (1933). *J. Physiol.* **79**, 255.
Drury and Szent-Györgyi (1929). *J. Physiol.* **68**, 213.
Euler and Gaddum (1931). *J. Physiol.* **72**, 74.
Frey and Kraut (1928). *Arch. exp. Path. Pharm.* **133**, 1.
Gavin, McHenry and Wilson (1933). *J. Physiol.* **79**, 234.
Guggenheim (1924). Die biogenen Amine, 2nd ed. (Berlin.)
Kraut, Frey and Werle (1930). *Z. physiol. Chem.* **189**, 97.
McHenry (1935). In press.