The Excretion of Inulin and Creatinine by the Human Kidney

A thesis

presented for the degree of Doctor of Philosophy of the University of Edinburgh

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INTRODUCTION

Historical - Theories Concerning Urine Formation.

The anatomical studies of Bowman (1842), in which he described the true relation of the glomerulus to the renal tubule, led him to suggest that the former removed water and inorganic salts from the plasma and that the other components of urine were added to this fluid by the renal tubules.

Two years later, Ludwig (1844) put forward an alternative theory suggesting that the glomerulus, under the influence of the hydrostatic pressure of the blood, formed a protein-free filtrate containing all the substances found in urine and that this filtrate was subsequently concentrated by the diffusion of water, and of some solutes, through the tubular cells back into the blood stream. This theory is now known as the filtration-reabsorption theory.

In 1874, Heidenhain modified Bowman's original theory suggesting that, although water and some salts were removed from the blood by the glomerulus, the renal tubules were chiefly responsible for the excretion of the urinary solutes. He produced evidence that the tubules of certain animals could excrete dyes independently of the glomeruli.

For the next fifty years these two theories, which had much in common - in particular the formation by the glomerulus of a filtrate of plasma, continued to be debated. It was not until Cushny (1917) produced his monograph 'The Secretion of Urine', summarising the then existing knowledge and postulating what is now known as the 'Modern Theory' of urine formation that renal

physiologists began to realise the correctness of Ludwig's original hypothesis.

Cushny considered that urine formation was composed of two discrete mechanisms, the formation by the glomerulus of an ultrafiltrate of plasma and the modification of this filtrate by reabsorption of water and solutes by the tubules. He considered that solutes were reabsorbed to a varying extent since the degree of concentration undergone in passing from plasma to urine varied from substance to substance. A substance which was filtered and completely reabsorbed, e.g. glucose, was termed a threshold substance, while substances which were not reabsorbed, e.g. the 'waste product' - creatinine, were termed non-thresholds. Cushny did not, however, believe that the renal tubules could excrete substances, a view currently held by most renal physiologists.

It remained for the micro-dissection studies of Richards and his co-workers (1938) to obtain direct evidence for many of the implications arising out of Cushny's theory.

The filtration-reabsorption theory, first put forward by Ludwig and now accepted by the majority of renal physiologists may be summarised:- The glomerulus forms, under the influence of the hydrostatic pressure of the plasma, a filtrate which contains all the substances in plasma which have molecular dimensions below a certain value - at present indefinable, but less than that of protein molecules. The passage of electrolytes from plasma to glomerular filtrate, however, is governed by the Donnan equilibrium. This filtrate passes along the tubules where it is modified by the tubular cells, actively by excretion or

reabsorption in the case of some solutes, and passively, by diffusion, in the case of others. This modification may take the form of reabsorption of the whole, e.g. glucose, or part, e.g. water or sodium ions, of a substance from the filtrate; or the addition of substances, e.g. hydrogen ions, ammonium ions and many foreign substances. The modified filtrate becomes eventually urine. There is also evidence to suggest that a substance may be both reabsorbed and excreted by the tubules under different conditions or even simultaneously, e.g. potassium, (Berliner, Kennedy and Hilton, 1950); thiosulphate, (Lambiotte, Blanchard and Graff, 1950).

The Measurement of the Glomerular Filtration Rate.

A first essential in the evaluation of the parts played by the glomerulus and the renal tubules in the elaboration of urine is the measurement of the rate of formation of filtrate by the glomerulus. This problem has attracted the attention of many workers in this field, and in the development of their studies the concept of renal clearance has done much to facilitate investigation.

This concept was first introduced by Moller, McIntosh and Van Slyke (1926), as a quantitative expression of the ability of the kidney to excrete urea. These workers defined clearance as the expression $\frac{UV}{P}$, where U is the concentration of the substance in the urine; V is the rate of urine flow; and P is the concentration of the substance in the plasma.

The originators of the term "clearance" regarded it purely

as a mathematical expression of one activity of the kidney and only some time later did its physiological significance become apparent. Using the above symbols, the amount of a substance excreted in the urine in a certain time, i.e. the rate of excretion, is expressed by UV, and this must equal the amount of the substance removed from the plasma in the same time, i.e. UV = CP, where C is the plasma clearance of the substance in question. The clearance is thus the volume of plasma which can be regarded as having been completely cleared of the substance in a certain time.

The term clearance was, at first, reserved for the excretion of urea but in 1931 Jolliffe and Smith extended the term to the excretion of creatinine and, subsequently, it has been extended to the excretion of other substances.

Rehberg, (1926) was the first to appreciate the fact that if a substance was neither reabsorbed, nor excreted, by the renal tubules then its clearance would form a measure of the glomerular filtration rate (GFR). Reasoning as above the volume of the plasma which will contain the amount of a substance which is excreted is given by the expression $\frac{UV}{P}$ and if this substance is neither reabsorbed nor excreted by the tubules, the amount excreted in a given time must equal the amount filtered. Since the process carried out by the glomerulus is simple filtration the concentration of a substance in the filtrate must be the same as the concentration in the plasma*. Thus the volume of plasma cleared

* More accurately the concentration in the filtrate equals the concentration in plasma water. Since however GFR is usually measured in terms of plasma it is generally unnecessary to introduce this refinement.

of the substance equals the volume of fluid filtered. In other words the GFR equals the clearance of this substance.

It follows from this that if a substance is excreted without tubular reabsorption or excretion, the rate of excretion is directly proportional to the plasma concentration, provided the GFR remains constant. This is an essential prerequisite for the acceptance of any substance which is to be used in the measurement of the glomerular filtration rate. It must be emphasised, however, that the converse to this statement does not necessarily hold good, since it is possible for a substance to be reabsorbed or excreted by the tubules in amounts proportional to the plasma concentration, so that this substance will satisfy the above criterion and yet its clearance will not equal the GFR.

It is necessary here to examine the effect of tubular activity on the excretion of a substance. If there is, as seems to be the case for most substances excreted or reabsorbed by the tubules, a limit to the amount that can be actively transported in a given time there will not be a direct proportionality between the rate of excretion, UV, and the plasma concentration, P. If a substance is reabsorbed, then at low plasma concentrations the amount excreted at any given plasma concentration will be less than the amount of a substance which is simply filtered. If the plasma concentration is increased the amount reabsorbed will reach a maximal value which will become small compared to the total amount filtered. The effect of tubular reabsorption will then become so small that the substance will appear to be filtered only and its clearance will approach the GFR. Its clearance will

therefore be relatively higher at higher plasma concentrations.

The converse is true for a substance which is secreted by the tubules. Its clearance at low plasma concentrations will be higher than the GFR; as the plasma concentration is increased the clearance will decrease so that it approaches the GFR. The clearance will therefore be relatively lower at higher plasma concentrations.

The presence of direct proportionality between the plasma concentration and the rate of excretion is a criterion which, above all, must be satisfied by a substance which is to be used in the measurement of the GFR. Several other criteria have been proposed but in man the above criterion is the only one which can satisfactorily be examined.

It is, perhaps, pertinent to summarise here these criteria.

1. The substance must be excreted at a rate which is directly proportional to the plasma concentration, provided that glomerular function remains constant. It may alternatively be stated that the clearance of the substance must be independent of the plasma concentration of the substance. If during the examination of this hypothesis by experiment the renal function does not remain constant, then the ratio of the clearance of the substance, at different plasma levels, to that of a second indifferent substance must be constant, provided that the excretion of the second substance is not itself dependent on other changing factors, e.g. urine flow.

2. The substance must be completely filterable through membranes not permeable to plasma proteins. Theoretically the

test should be made with the glomerular membrane itself, but this is, of course, impracticable. If a fraction of the substance in plasma is bound to protein or to some other non-diffusible substance then this fraction must be capable of accurate measurement.

3. When the clearance of the substance and that of one or more substances are identical under a number of different conditions then it is probable that all these substances are excreted solely by filtration and that the clearance of any one of them forms a measure of the GFR. This would be untrue if all the substances were excreted or reabsorbed by the tubules in exactly the same manner; for substances of different physical and chemical properties this would be most unlikely.

4. The clearance of substances which are known to be either reabsorbed or excreted by the tubules must approach that of the substance in question, as the plasma concentrations of these other substances are raised, provided that, as appears to be the case, there is a limit to the activity of the tubules in transporting these substances.

5. Similarly if the tubular excretion or reabsorption of a second substance is inhibited by some agent then the clearance of the second substance must approach that of the substance in question.

6. As presumptive evidence the substance should not be excreted by aglomerular kidneys such as are found in certain fish species, although difficulties arising from comparison between species are always large.

The Search for a Substance Suitable for the Measurement of Glomerular Filtration Rate in Man.

In 1926 Rehberg examined the excretion by the human kidney of a number of substances normally present in plasma. He found that of these creatinine was concentrated to the greatest extent, i.e. the ratio of its concentration in urine to the concentration in plasma was highest. Believing that tubular excretion did not normally take place, he concluded that creatinine was neither reabsorbed nor excreted by the renal tubules and that therefore it could be used to measure the GFR. Rehberg did not examine critically the relationship between the rate of excretion and the plasma concentration and because of analytical difficulties involved in the measurement of the normal creatinine concentration in plasma, he raised the plasma creatinine level by oral administration of creatinine.

During the years between 1926 and 1935 a number of workers examined the excretion of creatinine at various plasma levels and their results seemed to indicate that the rate of excretion was proportional to the plasma concentration. However analytical difficulties, especially at concentrations near to the endogenous level, made interpretation of their results difficult.

In 1935, Shannon examined the relationship between the rate of excretion (UV) and the plasma concentration of creatinine (P) over a wide range of plasma concentrations, from 10 to 150 mg./ 100 ml. and found that over this range UV was not proportional to P. He concluded from this that creatinine was excreted by the

tubules and that its clearance was therefore an overestimate of the GFR.

In the same year Shannon and Smith (1935) suggested the use of the polysaccharide inulin in place of creatinine. They did so on experimental evidence that in man inulin was excreted at a rate proportional to the plasma concentration and that in phlorizinised man the simultaneous clearances of inulin and xylose were approximately equal. In addition there was evidence that inulin was not excreted by aglomerular kidneys.

Subsequently other groups of workers (Miller, Alving and Rubin, 1939; Josephson and Lindahl, 1943; Hogeman, 1943; and Ahlborg, 1947) examined further the excretion of inulin and reported findings essentially in agreement with those of Shannon and Smith, so that the evidence put forward by the latter rejecting creatinine as a substance suitable for the measurement of the GFR and that in favour of inulin was generally accepted as conclusive.

In 1950, however, Ferguson, Olbrich, Robson and Stewart presented data showing that the inulin clearance was dependent on the plasma concentration and pointed out that the evidence in support of the use of inulin was less conclusive than was generally believed. Furthermore, in addition to this evidence casting doubt on the validity of inulin clearance as a measure of the GFR there was forthcoming at this time evidence to refute Shannon's conclusion that creatinine clearance was dependent on the plasma concentration, for Hare (1950) reported that at low plasma concentrations the creatinine clearance was the same as at high plasma concentrations.

Apart from inulin and creatinine few other substances have been put forward as being suitable for use in the measurement of the GFR in man.

Thiosulphate was originally reported to have a clearance identical with that of inulin, (Newman, Gilman and Phillips, 1946), but subsequently evidence has been put forward that thiosulphate may under some circumstances be excreted by the tubules (Lambiotte et al., 1950).

Allantoin was reported by Friedman, Byers and Abrahm, (1947) to have a clearance identical to that of inulin. In patients with renal pathology, however, the allantoin clearance has been reported to be considerably higher than the inulin clearance, (Miller, Leaf, Mamby and Miller, 1952b).

A value of 0.99 has been reported for the mean sucrose/ inulin clearance ratio (Steinitz, 1940) but this has not been confirmed.

All of these three substances have, thus, been rejected on the grounds that their clearances are not identical with the inulin clearance under all circumstances. However, this would not be a valid reason for rejection if in fact it was ~~essential~~ established that inulin does not satisfy the criteria listed above.

The investigations presented in this thesis were undertaken in an attempt to elucidate further the mechanisms of the renal excretion of inulin and creatinine in man and to establish the mutual relationships between the glomerular filtration rate and the renal clearances of inulin and creatinine. For the purpose of this thesis the investigations have been considered in three

sections.

1. The excretion of inulin including observations on the determination of inulin in serum and urine.

2. The determination of creatinine in serum and urine.

3. The excretion of creatinine and the relationship between the excretion of inulin and creatinine.

I. THE EXCRETION OF INULIN IN MAN

The Properties of Inulin.

a) Chemistry.

Inulin is a polysaccharide which was first isolated from artichoke tubers by Rose (1804) and was given the name inulin by Thomson (1811). It occurs principally in the tubers of certain plants of the Compositae family, in particular in the Jerusalem artichoke, dahlia and chichory. Although it is composed mainly of fructose units several workers have reported the presence of d-glucose in the product of hydrolysis brought about by mineral acids or purified enzymes, (Schlubach and Elsner, 1929; Jackson and MacDonald, 1930; Adams, Richtmyer and Hudson, 1943; and Palmer, 1951).

The solubility in water varies with the temperature and with the method and source of preparation (Lippman, 1904; Yanovsky and Kingsbury, 1933). When recrystalised from water its solubility is much less than when it was precipitated from aqueous solution by alcohol. A 20% solution can be prepared by dissolving inulin in water at 80°C. and, on cooling to 37°C., a supersaturated solution is obtained which is stable for several hours.

Its molecular weight has been variously reported to have a value from 312 to 8,000, though the majority of figures reported lie in the range 5,000 to 8,000 (Haworth, Hirst and Percival, 1932; Drew and Haworth, 1928; Westfall and Landis, 1936; and Palmer, 1951).

The wide range reported is probably a reflection of the

difficulty of obtaining an ash-free sample and with the readiness with which it apparently assumes different molecular forms.

In biological fluids inulin is amenable to accurate chemical analysis, the existing methods being based either on the colour reaction with diphenylamine or on the colour reaction with resorcinol after hydrolysis by mineral acid. Glucose interferes in these methods to a small extent and this substance together with free fructose and other unknown substances in normal plasma, and to a smaller extent in normal urine, give rise to an inulinoid blank. Treatment of test solutions, e.g. plasma or diluted urine, with yeast has been advocated to reduce the interference from glucose and fructose, but after yeasting a small inulinoid blank still remains. It is assumed that during the course of experiments involving administration and determination of inulin in plasma and urine the inulinoid blank determined at the commencement of the experiment remains constant throughout its course.

b) Physiology.

Inulin is physiologically inert and non-toxic in doses required for physiological investigations (Smith, Chasis and Ranges, 1938; Smith, 1951). Its intravenous administration in man produces no detectable changes in renal function, or in the circulatory or other systems even in doses of as much as 160 g. (Smith, 1951). There are no enzymes in blood capable of hydrolysing it (Smith, 1951) and after intravenous administration it is rapidly and quantitatively excreted in the urine (Shannon and Smith, 1935; Josephson and Lindahl, 1943; Smith, Finkelstein

and Smith, 1940; Gaudino, Scwartz and Levitt, 1948). It does not enter erythrocytes (Richards, Westfall and Bott, 1934, Smith, 1937). After prolonged intravenous infusion it assumes a volume of distribution in man of 15-16% of the body weight, a fraction corresponding to what is believed to be the extracellular fluid volume. Its volume of distribution is less than that of radioactive sodium (Na^{24}), thiocyanate or bromide ions (Gaudino, Schwartz and Levitt, 1948); and less than that of mannitol (Schwartz, Breed and Maxwell, 1950; it is equal to that of sucrose (Deane, Schreiner and Robertson, 1951).

Inulin possesses an elongate molecule which gives it an effective diffusion coefficient equivalent to that of a substance with a molecular weight about 15,000 (Bunim, Smith and Smith, 1937). Its diffusion coefficient is therefore about twice that of the haemoglobin molecule and about a fifth of that of creatinine. It is completely filterable from human or dog plasma through collodion membrane (Shannon and Smith, 1935). The low diffusibility of inulin compared to substances with smaller molecular sizes, e.g. thiocyanate ion, sodium ion, creatinine, mannitol and sucrose, results in a longer period being required for the attainment of a uniform concentration throughout the volume of distribution, after the start of a continuous infusion (Gaudino and Levitt, 1949). The time required for the attainment of equilibrium in man is about 6 hr. compared to a time of 1 hr. for sucrose (Gaudino, Schwartz and Levitt, 1948). This is an important finding especially in its effect on the kinetics of inulin distribution following a single injection. Robson,

Ferguson, Olbrich and Stewart, (1949) and Schater, Freinkel and Schwartz (1950) have pointed out that after a single injection of inulin, equilibrium between the plasma and the extravascular compartment of the extracellular fluid is never, in fact, reached.

The Renal Excretion of Inulin in Man.

Shannon and Smith (1935) were prompted to examine the excretion of inulin in man for the following reasons.

1. Inulin is not excreted by the aglomerular fish Lophius and Opsanus (Richards, Westfall and Bott, 1934; Shannon, 1934). In this it resembles other carbohydrates, e.g. glucose, lactose, xylose and sucrose (Marshall, 1930, 1934; Jollife, 1930; Clarke and Smith, 1932; Smith, 1935).

2. In the dogfish, which has glomeruli, the xylose clearance is lower than the simultaneous inulin clearance by about 20% while in the phlorizinised dogfish the clearances are equal (Shannon, 1934).

3. In the frog, when inulin is injected into the general circulation, it appears in the glomerular fluid in the same concentration as it is in plasma; however it does not appear in the urine when it is injected into the renal portal system where it comes in contact only with the renal tubules (provided there has been no recirculation) (Hendrix, Westfall and Richards, 1934).

4. Xylose and sucrose clearances in man differ by a few per cent only and the clearances of these two substances become equal and also equal to the clearance of glucose in phlorizinised

man (Keith, Power and Peterson, 1933; Chasis, Jolliffe and Smith, 1937).

Shannon and Smith administered inulin to four normal subjects by an intravenous infusion and measured the inulin clearance after the end of the infusion while the plasma concentration of inulin was decreasing. Clearances were calculated using the plasma concentration at the mid-point of the clearance period. They examined their data by plotting the rate of excretion of inulin (UV) against the plasma concentration (P). They constructed the best fitted line to their experimentally determined points apparently by visual inspection and noted that it passed through the zero coordinates in all four cases. They concluded from this that inulin was excreted at a rate which was proportional to the plasma concentration, i.e. that its clearance was independent of the plasma concentration and, therefore, a measure of the GFR. It should be noted, however, that by the use of relatively high concentrations of inulin, viz. 50-400 mg./100 ml., the effect, which the reabsorption of a small amount of inulin would produce, would be completely obscured.

A summary of the findings of Shannon and Smith relevant to the relationship between the plasma concentration and the rate of excretion of inulin, together with those of later investigators, is given in Table 1.

Shannon and Smith also reported that the xylose clearance in man was about 20% lower than the inulin clearance. After the administration of phlorizin, a substance which tends to lower all clearances due to its effect on the circulatory system, the

TABLE 1. Relationship between Clearance and Plasma Inulin Concentration - Summary of Literature.

Author	Subjects	Procedure	Plasma Levels	Results
Shannon & Smith 1935	4 normals	Single infusion. Started collections 30 min. after the end of infusion	50 - 400	Clearance (Cin), independent of plasma concentration (P)
Miller et al. 1940	5 normals & 7 with renal pathology	a) Single injection. Started 30 min. after injection	5 - 40	Cin independent of P.
		b) Continuous infusion. Started 30 min. after prime	5 - 100	Cin at high P slightly higher than at low P.
Josephson & Lindahl, 1943	25 normals	Single injection. Started 60 min. after injection	not stated	1st hr. Mean Cin 137 ml./min 2nd " " " 143 " " 3rd " " " 117 " "
Hogeman, 1943	35 normals	Single injection. Started 60 min. after injection.	not stated	1st 20 min. Cin 120 ml/min. 2nd " " " 119 " 3rd " " " 121 "
Ahlborg, 1947	15 normals	Single injection. Started 60 min. after injection.	not stated	1st 30 min. Mean Cin 126 ml/min 2nd " " " " 117 ml/min 3rd " " " " 121 ml/min
Ferguson et al. 1949	15 normals & 3 hypertensives	a) Single injection. Started 30 min. after injection	10 - 60	Progressive fall in Cin with fall in P.
		b) Continuous infusion	15 - 100	Cin at high P higher than Cin at low P.
Mattar et al. 1952	3 children with nephrotic syndrome	Continuous infusion. Three levels	15 - 200	Cin independent of P.
Kennedy & Kleh 1952	14 normals	Continuous infusion. Three levels	5 - 175	Cin independent of P.
Mandel et al.,	- normals	Continuous infusion. Two levels	10 - 90	Cin independent of P.

xylose clearance was only 10% lower than the inulin clearance. They considered that this was evidence that xylose was normally partly reabsorbed by the tubules, a process inhibited by phlorizin. The remaining discrepancy between the clearances was considered to be due to back diffusion of the smaller xylose molecules.

The approximate equality of the clearances of xylose and inulin after the administration of phlorizin was considered as further evidence that inulin was excreted solely by glomerular filtration. They did, however, admit that their data did not exclude the possibility of the reabsorption of a small amount of inulin. In view of what one of the authors has subsequently to say about clearances determined while the plasma concentration is changing (Smith, 1951), it is, perhaps, surprising to note that the original data regarding the excretion of inulin on which so much has subsequently been based was obtained, in fact, from experiments in which the plasma concentration of inulin was continuously decreasing.

No further systematic examination of the influence of plasma concentration on the clearance of inulin was reported until 1939 when Miller, Alving and Rubin re-investigated this question. Because of the relative inaccuracy and non-specificity of the analytical procedure available to them (difference in the reducing power before and after hydrolysis) for the estimation of inulin, Shannon and Smith were forced to use relatively high plasma concentrations of inulin. By 1939, however, there were available several more specific and more sensitive methods and Miller et al. pointed out that if a small amount of inulin was reabsorbed by the tubules this would become more apparent at lower plasma concentrations

provided the tubules exhibited a maximum in their ability to reabsorb inulin as they had been shown to exhibit in their ability to reabsorb other substances, e.g. glucose.

Miller et al. measured inulin clearances at two plasma concentration levels in the range 4-80 mg./100 ml. using continuous infusions of inulin to maintain plasma concentrations of inulin constant in some experiments and single injections of inulin in others. Their data referring to inulin clearances determined in subjects receiving continuous infusions has been summarised in Table 2. The inulin clearances determined at the lower plasma concentrations have been expressed as a percentage of the clearance at the higher plasma concentration in order to allow the comparison of different absolute clearances. The mean clearance at the lower plasma concentrations, expressed as a percentage of the higher plasma concentration, has been calculated for the whole series and separately for the six experiments carried out on normal subjects or hypertensive subjects with normal renal function (C_{in} > 80 ml./min.). It will be noted that while the mean percentage clearance is in neither case significantly different from 100% (p less than 0.2 and p less than 0.8 respectively), seven out of nine of the subjects have a lower inulin clearance at the lower plasma concentration.

In the five experiments where a single injection of inulin was given there was no consistent change in inulin clearance with change in plasma concentration. In one experiment the inulin clearance decreased with decreasing plasma concentration; in another it increased with decreasing plasma concentration and in

TABLE 2. A. Data of Alving et al. (1940). Comparison of inulin clearances at different plasma concentrations obtained with continuous infusions of inulin.

Subject	Mean inulin clearance at highest P.	Mean inulin clearance at lowest P.	C_{in} at low P. % of C_{in} at high P
	ml./min.	ml./min.	%
DK.	125	111	87
MB.	128	114	89
BE.	142	121	85
BH.	111	115	104
IM.	110	86	78
VS.	104	87	83
ES.	67	58	86
AT.	13.0	17.6	135
MC.	22.0	22.6	94

B. Significance of difference between clearances at high and low P.

Subject	No.	Mean % clearance (lowP/high P)	Significance of difference*
all	9	87.6	P 0.2
with C_{in} in normal range (80 ml./min).	6	93.4	P 0.8

* Calculated by Student's t test. To be significant p must be less than 0.05.

the remaining three it remained virtually constant.

Miller et al. concluded that in most of their experiments the inulin clearance was independent of the plasma concentration, although they did admit the possibility of the reabsorption of small amounts of inulin, - 1-2 mg./min., in a few individual clearance periods. However, as Ferguson et al. have pointed out, the amount of inulin which was possibly reabsorbed has been calculated for plasma concentrations in the region of 5 mg./100 ml. and, if tubular reabsorption of inulin occurs, the amount reabsorbed will be maximal at high plasma concentrations and may therefore be greater than the estimates given by the authors.

In 1943, Josephson and Lindahl measured inulin clearances in 25 normal persons by giving a single intravenous injection of inulin and measuring clearances over three hourly periods starting 60 min. after the end of the injection. The mean inulin clearances obtained were as follows:-

Period 1. Mean inulin clearance 137 ml./min.

Period 2. " " " 143 ml./min.

Period 3. " " " 117 ml./min.

The difference between the mean clearances in the first and second periods was not statistically significant but the difference between the values for the second and third periods was statistically significant. Since the plasma inulin concentrations were decreasing during the experiment the changes observed in clearance correspond to changes in P.

The authors were unable to offer any explanation for the fall in mean clearance during the third period. Since there was

no significant change during two hours, while P was continuously decreasing, the results suggest that inulin clearance was independent of P over a wide range of the latter. Three to four hours after the injection of 5g. inulin the plasma inulin concentration would have been of such a magnitude that small errors in the determination of the total inulin concentration and in the inulinoid blank might conceivably been responsible for the results obtained.

In the same year Hogeman (1943) also reported the results of inulin clearance determinations carried out on 35 normal subjects using a single injection technique. Starting 60 min. after the end of the injection the inulin clearances were measured during three 20-minute periods. The mean inulin clearances obtained were as follows:-

Period 1. Mean inulin clearance 120 ml./min.
Period 2. " " " 119 ml./min.
Period 3. " " " 121 ml./min.

These results also suggest that the inulin clearance was independent of the plasma concentration during the time, 60 - 120 min. after the injection, in which they were measured.

Ahlborg (1947) measured inulin clearance in 15 normal persons also using a single injection technique. Starting 60 min. after the injection he measured inulin clearances during three 30-minute periods. His results were very similar to those of Hogeman, viz.:-

Period 1. Mean inulin clearance 126 ml./min.
Period 2. " " " 117 ml./min.
Period 3. " " " 121 ml./min.

Again these results suggest that inulin clearance was independent of the plasma concentration over the range studied.

In 1950, Ferguson, Olbrich, Robson and Stewart reported the results of inulin clearance determinations in 15 subjects given single intravenous injections of inulin and in three subjects in whom the plasma inulin concentration was maintained approximately constant at two or three different levels by a continuous infusion of inulin. Clearance determinations were made from 15 - 150 min. after the injection of inulin.

In the single injection experiments the inulin clearances were calculated by dividing the mean rate of inulin excretion by the estimated mean plasma concentration for that period, this latter being read from a smooth curve drawn through the experimentally determined plasma inulin concentrations plotted against time. A delay-time of 2.5 min., or alternatively 6 min., was allowed in estimating the plasma concentration. In the continuous infusion experiments the mean rate of inulin excretion was divided by the average plasma concentrations at the beginning and end of the period. In both series it was noted that there was a tendency for the inulin clearances to decrease as the plasma inulin concentration decreased though this tendency was much more marked in the single injection series.

The results of the single injection series were analysed further by plotting UV against P and calculating the deviation of

the linear regression line, through these points, from the zero coordinates. In every case this intercept on the UV axis was negative and was statistically significant in 12 experiments out of a total of 15 when a delay-time of 2.5 min. was allowed and in 10 experiments when a delay-time of 6 min. was allowed.

In the continuous infusion experiments the mean rate of excretion was plotted against the mean plasma concentration measured at the highest and lowest plasma concentrations and it was found that the line calculated to pass through these points had negative intercepts on the UV axis of the same order of magnitude as in the single injection experiments.

In order to exclude the possibility of error due to the sampling of venous blood the arterio-venous difference in plasma inulin concentration was determined in 5 subjects after they had been given a single injection of inulin. The mean difference for each subject varied from -3% to +1%, although in individual instances the difference was as much as -11% to +4%. The mean A-V difference found was less than that found by Brun, Hilden and Raschou (1950) who found a mean A-V difference of 7.4%. Even this, however, would not account for the results obtained.

Ferguson et al. concluded that their results strongly suggested that inulin is reabsorbed by the tubules to a significant extent. In considering the results obtained by earlier workers, they point out that Shannon and Smith used such high plasma inulin concentrations that the effect of the reabsorption of a small amount of inulin would be entirely obscured in their

data and, further, that the results of the continuous infusion experiments of Alving et al. do, in fact, show a tendency for the clearance of inulin at high plasma concentrations to be greater than the clearance at low plasma concentrations. They do not, however, offer any explanation for the discrepancy between the results of their single injection experiments and those carried out by Alving et al., Hogeman, and Ahlborg where no consistent dependence of inulin clearance on plasma concentration was observed.

This then comprises a review of the relevant literature on this subject.*

In view of the significance which has generally been attached to the inulin clearance as a measurement of the glomerular filtration rate in man it seemed most important to try and resolve the problem created by the apparently inconsistent findings of those investigators who had studied the excretion of inulin.

*Since this investigation was started reports of three further investigations on the subject of inulin clearance at different plasma concentrations have been published. Of these the most important is that of Kennedy and Kleh (1953) who, using a continuous infusion of inulin determined inulin clearances at three different levels of plasma concentrations (P_{in} = 5-175 mg./100 ml.) in 14 normal subjects. They were unable to show any dependence of inulin clearance on plasma concentration.

Mandel, Cargil and Jones (1953), also reported that inulin clearance was independent of plasma concentration in continuous infusion experiments.

Mattar, Barett, McNamarra and Lauson (1952), who also used continuous infusion techniques, reported that in three children with nephrotic syndrome the inulin clearance was independent of the plasma concentration.

In particular it seemed important to try and obtain evidence which would decide whether inulin clearance could be rejected as a valid measure of the glomerular filtration rate in man. Since most of the evidence presented by Ferguson et al. was obtained from single injection experiments and since difficulties in interpreting clearance data obtained from experiments where the plasma concentration is not constant are generally believed to exist, it was decided to investigate further the relationship between the plasma concentration of inulin and the inulin clearance using continuous infusion experiments only. A few preliminary experiments were carried out on the determination of inulin and on the homogeneity of the inulin preparations used. These are described first.

Experimental.

I. The Estimation of Inulin in Serum and Urine.

The method used for the estimation of inulin was essentially Cole's modification of that of Steinitz (1938). This is a colorimetric method based on the production of an orange colour when inulin is heated with resorcinol in the presence of hydrochloric acid and ferric chloride. The procedure finally adopted is given first followed by an account of some experiments concerning this method.

Reagents

Inulin Kerfoot and Sons.

Standard solutions were made by dissolving 100 mg. in about

20 ml. water at 80°C., cooling the solution, transferring the solution to a 100 ml. volumetric flask and making up to 100 ml. with water. The actual standards containing 1.0 - 4.0 mg./100 ml. were made by further dilution.

Resorcinol 200 mg./100 ml. in ethanol.

This solution was prepared daily. The ethanol was purified either by the silver nitrate/sodium hydroxide method or by the diphenylamine method.

Ferric chloride. (A.R.) 1.25 mg./100 ml. in concentrated hydrochloric acid (A.R., s.g. 1.18).

Zinc sulphate (A.R.) 12.5 g. $ZnSO_4.7H_2O$ were dissolved in 125 ml. 0.25N H_2SO_4 and made up to 1,000 ml. with water.

Sodium hydroxide (A.R.) 0.75 N.

When 50 ml. of the zinc sulphate solution were titrated with the sodium hydroxide solution using phenolphthalein as indicator a volume of 6.7 - 6.8 ml. was required.

Absorptiometer Unicam S.P. 500, or
Unicam S.R. 600.

Methods

Serum - precipitation of serum proteins.

1.0 ml. serum, 8.0 ml. zinc sulphate solution and 1.0 ml. of sodium hydroxide solution were added to 100 ml. conical flask and the contents of the flask mixed by rotation. The mixture was allowed to stand for 30 min. and then filtered through a Whatman No. 1 filter paper. This gave a filtrate equivalent to serum diluted 1 in 10. This filtrate was further diluted, when

necessary, to give an inulin concentration of between 1.0 and 4.0 mg./100 ml.

Urine. If protein was present the urine was treated as serum. Otherwise a suitable dilution was made with water to give an inulin concentration of between 1.0 and 4.0 mg./100 ml.

Into a test tube fitted with a B19 ground-glass stopper were pipetted 3.0 ml. serum filtrate, diluted urine or standard inulin solution, 2.0 ml. resorcinol and 3.0 ml. ferric chloride in that order. The tubes were then stoppered and the contents mixed. The stoppers were then loosened and the tubes put into a water bath maintained at 80 ± 0.2°C. After two minutes the stoppers were again tightened and the tubes were then incubated for a total of 15 min. The tubes were then placed in a mixture of ice and water to stop the reaction. After 5 min. the tubes were removed from the ice-water and were then left at room temperature for 15 min. The contents of the tubes were then mixed again and the density of the developed colour was measured in either of the Unicam absorptiometers at a wavelength of 480 mμ, using a 10 mm. cuvette. The colour was found to be stable for 2 hr.

A standard curve was constructed for each batch of tubes by plotting the concentration of standard solutions of inulin, which were always included, against the optical density. The concentration of the unknown solutions were obtained from this curve.

Observations on the Method.

In adapting the method of Cole for use with either the Unicam S.P. 500 or Unicam S.P. 600 it was necessary to determine the optimum wavelength at which to measure the optical densities. The absorption spectrum of the developed colour exhibits a broad peak with a maximum in the region of 480 mμ (Fig. 1). Determinations were carried out, therefore, using this wavelength.

The colour developed during incubation did not reach a maximum intensity until after thirty minutes (Fig. 2). By this time the pure orange colour had become suffused with a dusky colour which was probably the result of charring. For this reason it was considered that there was no advantage to be gained by using an incubation time longer than that recommended by Cole, i.e. 15 min. The colour which developed in this time did not obey Beer's Law, (Fig. 3), although the deviation from it was not great.

The rate of colour development was found to depend rather critically on a number of variables including the concentrations of the reagents, and the temperature and time of incubation and in view of this standard curves were constructed for each batch of unknowns by including standard inulin solution with each batch. The protein precipitating reagents were found to give the same colour as a water reagent blank so that the colours developed from serum filtrates were read against the latter.

Determinations in Serum and Urine.

With the above method, as has been reported for most methods

which have been used for the estimation of inulin, normal plasma or serum was found to contain material which gave a similar orange colour. This has been termed the inulinoid blank.

Glucose subjected to the above procedure gave very much less colour than did inulin (Fig. 4). A solution of glucose containing 100 mg./100 ml. (diluted 1 in 10 as in protein precipitation) gave the same colour as was calculated to be given by a solution of inulin containing 0.6 mg./100 ml. (diluted 1 in 10). Bacon and Bell (1946) using essentially the same method reported that glucose gave 0.6 - 0.8% of the colour given by fructose.

Free fructose, which has been reported to be present in plasma (Bacon and Bell, 1948; Hubbard and Russell, 1937; Mann, 1946; Wallenfals, 1951), no doubt contributes to the inulinoid blank. However yeasting the plasma does not remove all the inulinoid blank so that substances other than glucose and fructose must be responsible for some of the colour.

The inulinoid blank in serum was found to be increased after meals. In all studies on the renal excretion of inulin a serum inulinoid blank was determined on a sample of serum obtained before the inulin was administered and the inulin equivalent was subtracted from the total inulin concentrations determined in sera obtained during the experiment. The error involved in neglecting the inulinoid blank is not significant at serum inulin concentrations in the region of 50 mg./100 ml. but at lower concentrations becomes increasingly important. In applying this correction it was assumed that the inulinoid blank remained constant throughout the experiment. There was no means of confirming this, but as the inulin

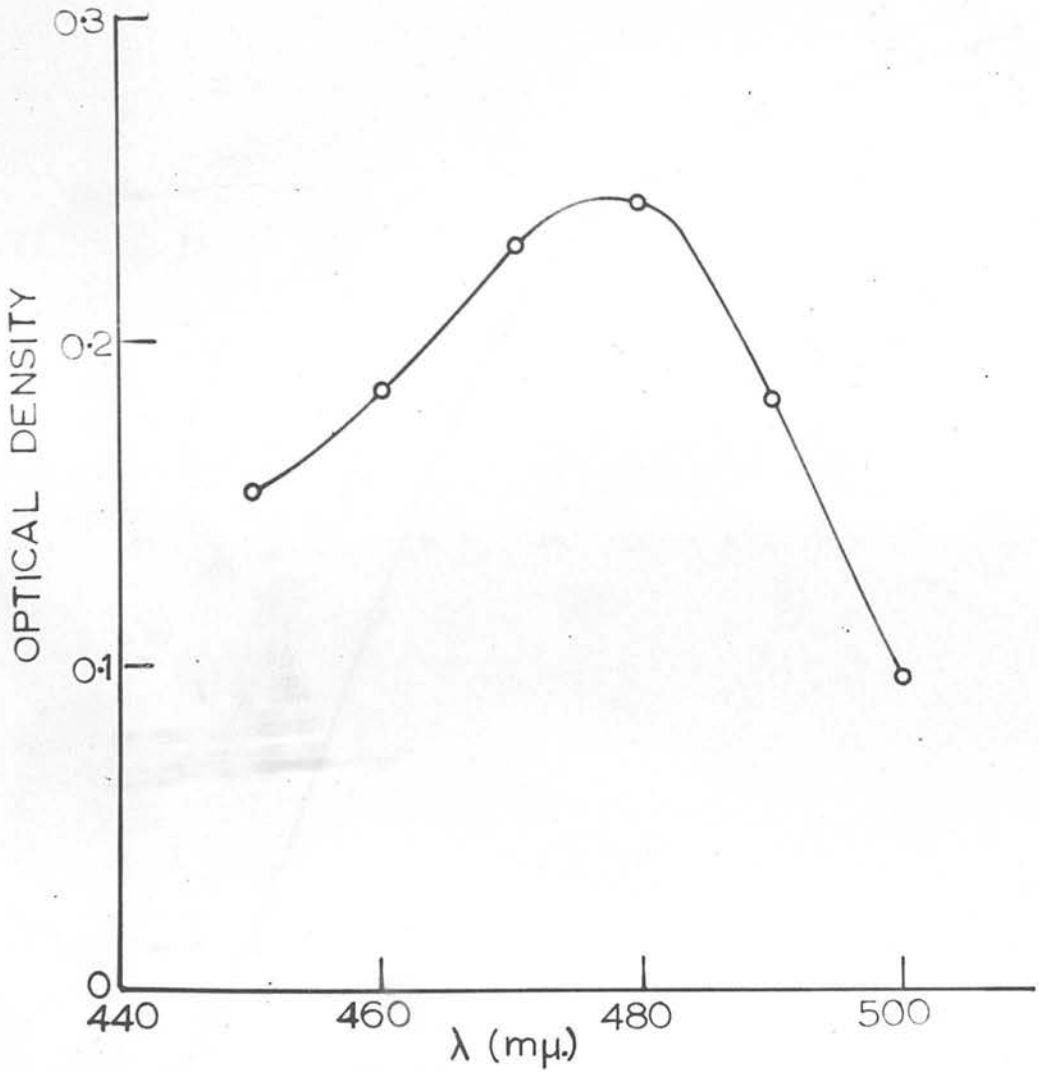

FIGURE 1. Inulin - resorcinol/ferric chloride reaction. Absorption spectrum.

Inulin (2.0 mg./100 ml.) 3 ml., resorcinol 2 ml., HCl-$FeCl_3$ 3 ml.; Incubated 80°C. for 15 min. 10 mm. cell

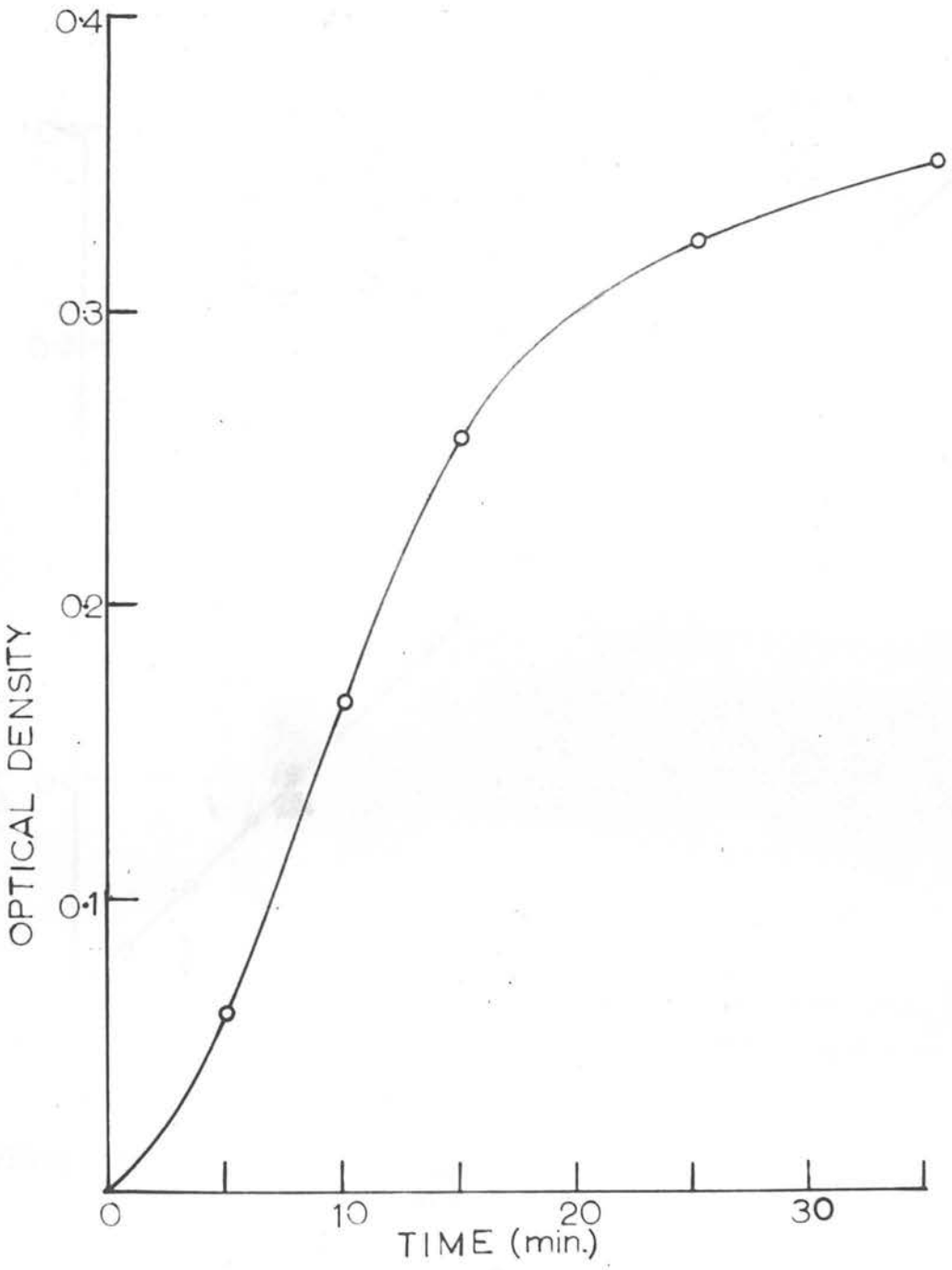

FIGURE 2. Inulin - resorcinol/ferric chloride reaction. Rate of colour development.

Inulin (2.0 mg./100 ml.) 3 ml., resorcinol 2 ml., HCl-$FeCl_3$ 3 ml.; Incubated 80°C. for various times; 480 mμ; 10 mm. cells.

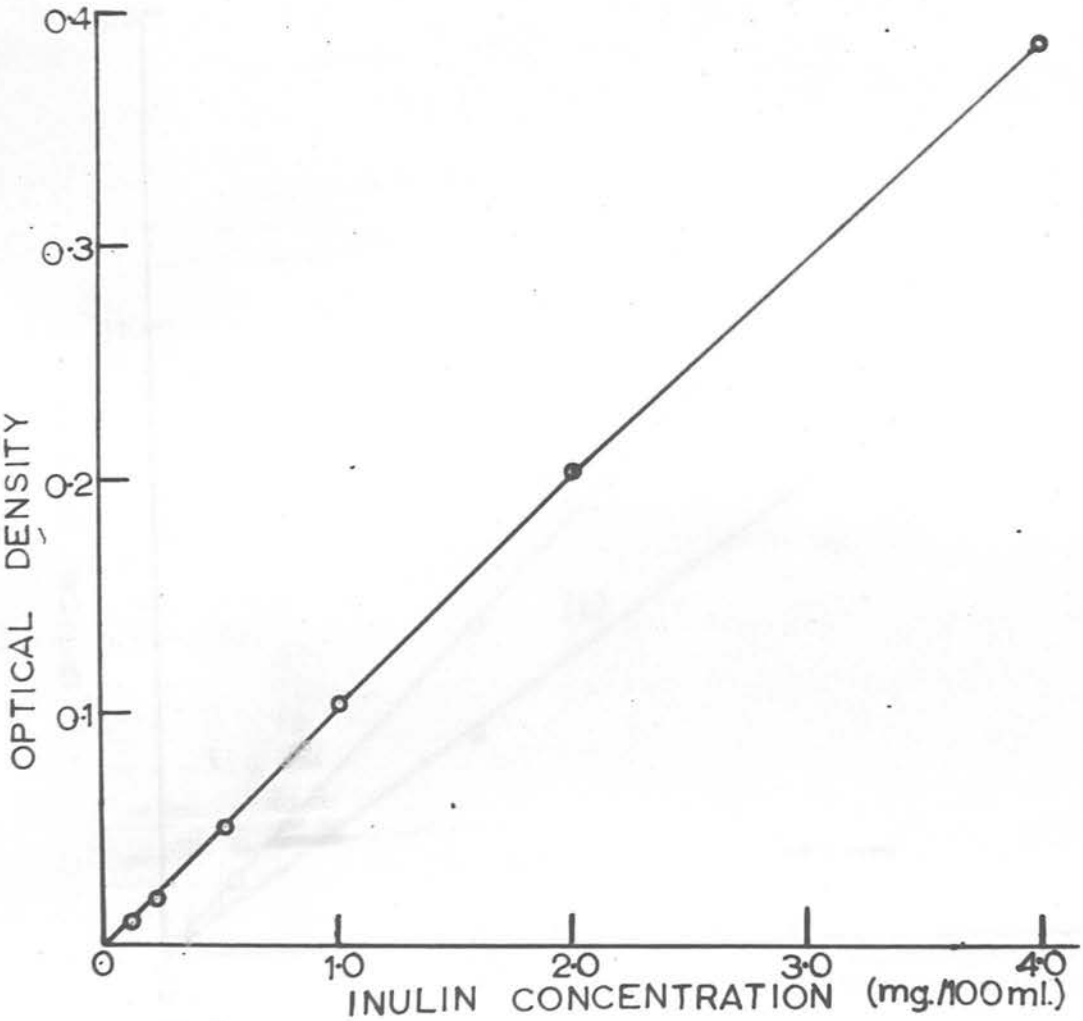

FIGURE 3. Inulin - resorcinol/ferric chloride reaction. Optical density and inulin concentration.

Inulin 3 ml., resorcinol 2 ml., HCl-$FeCl_3$ 3 ml.; Incubated 80°C. for 15 min.; 10 mm. cells.

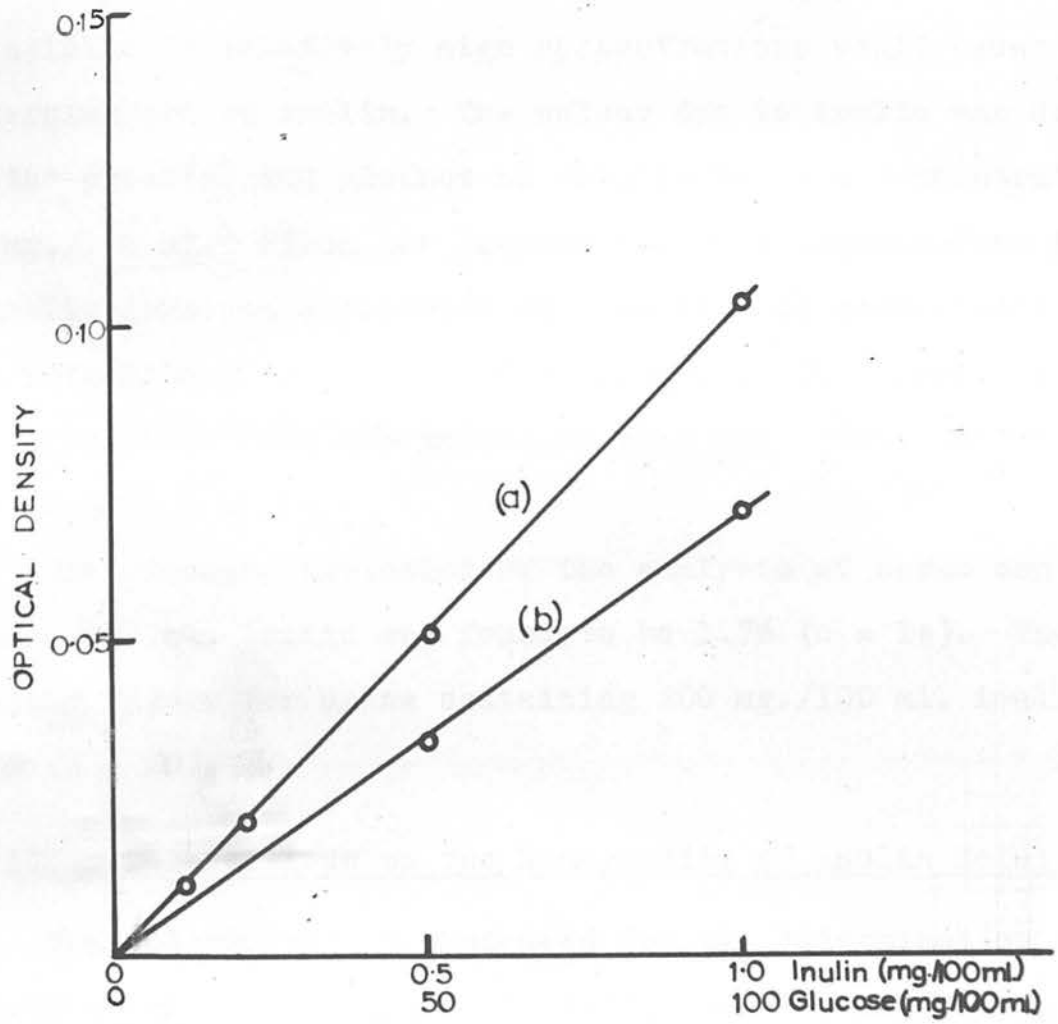

FIGURE 4. The interference by glucose with the determination of inulin.

Inulin, or glucose 3 ml., resorcinol 2 ml., HCl-$FeCl_3$ 3 ml. Incubated 80°C. for 15 min.; 480 mμ.; 10 mm. cells.

was administered only to subjects in the post-absorptive state it was considered that no significant error would be introduced by this assumption.

In some of the experiments on the excretion of inulin creatinine was administered simultaneously. It was therefore necessary to determine what interference, if any, the presence of creatinine in relatively high concentrations would cause in the determination of inulin. The colour due to inulin was developed in the presence and absence of creatinine at a concentration of 10 mg./100 ml. Since the preparation of a protein-free filtrate normally involved a dilution of 1 in 10 this corresponded to a concentration in serum of 100 mg./100 ml. The results show that the creatinine does not interfere with the determination of inulin (Table 3).

The standard deviation of the analysis of serum containing 20 mg./100 ml. inulin was found to be 1.7% (n = 14). The corresponding figure for urine containing 200 mg./100 ml. inulin was 1.4% (n = 20).

II. Observations on the Homogeneity of Inulin Solutions.

The colorimetric method used for the determination of inulin measures the total fructose present, i.e. the fructose present in the inulin molecules, which is hydrolysed rapidly under the conditions employed, and in any smaller molecules composed of fructose units, including free fructose molecules. If a solution containing inulin and fructose molecules was administered intravenously, determination of the inulin concentration in the serum by the above

TABLE 3. Effect of Creatinine on Determination of Inulin

Creatinine concentration	0.0 mg./100 ml.	100 mg./100 ml.
Inulin concentration	Optical density	Optical density
mg./100 ml.		
0	.027	.028
20	.289	.291
40	.546	.547

method would give erroneously high results. Since the renal clearance of fructose is much less than that of inulin due to the reabsorption of fructose molecules (Gammeltoft and Klerilf-Jansen, 1943) the use of such a preparation of inulin would cause the apparent inulin clearance, calculated from total fructose concentrations in plasma and urine, to be lower than the true inulin clearance. It is possible, also, that small fragments of the inulin molecule have a clearance less than that of inulin due to partial reabsorption so that even if there were present molecules composed of several fructose units, i.e. partial fragmentation of the inulin molecule, the apparent clearances obtained would be lower than the true inulin clearance.

In the preparation of inulin for administration, it was heated in the appropriate amount of 0.9% (w/v) sodium chloride solution to a temperature of just below 100°C. in order to dissolve the inulin and the resulting solution was filtered through an autoclaved Seitz filter. In the preparation of large volumes of inulin solution, owing to the small capacity of the Seitz filter the inulin solution could not all be poured into the filter at once so that the unfiltered portion was either kept hot - at a temperature of above 70°C., or was brought to the boil at intervals until all of it could be poured into the filter. (This was an unnecessary procedure, as was discovered later, since the supersaturated solution did not tend to precipitate inulin in passing through the filter even when the temperature was much lower).

Inulin was reported by MacDonald (1946) to undergo apparent

decomposition in hot solution. If the method of preparation of the inulin for administration brought about a partial decomposition of the inulin molecules then the effect of the smaller fragments on the determination of the inulin clearance might be that already described.

Josephson and Lindahl (1943), reported that, using some preparations of inulin, the inulin clearance was much lower than with others, and in addition, the recovery of the injected inulin in the urine was much lower. (Normally this is in the region of 100%). One insulin preparation which gave anomalous results was made by subjecting the inulin solution to a temperature of 100°C. for three 20-minute periods.

In an attempt to evaluate the error which might be caused by the presence of small fragments of the inulin present in the original sample or produced during the preparation of the inulin solution for administration, two series of experiments were carried out.

1. The determination of the yeast-fermentable material present in the inulin solution, before and after heating for various times.

2. The determination of the changes in total reducing power of the solution produced by heating for various times as a measure of the decomposition of the inulin molecules.

Experiments with Yeast.

Inulin is only slowly hydrolysed by yeast (Adams, 1942), whereas fructose is rapidly metabolised. If a solution of inulin is subjected to the action of yeast, any decrease in the total

fructose concentration indicates the presence of fructose molecules or of small fragments of inulin molecules which might be expected to be metabolised by yeast though at a slower rate than fructose itself.

A suspension of yeast was prepared by washing a sample of bakers' yeast twice with five times its volume of water and suspending it in its own volume of water. The exact concentration of yeast was determined by centrifuging a sample of the suspension in a haematocrit tube. A mixture of 4.0 ml. of an inulin solution containing 20 mg./100 ml., prepared as for standard solution, and 1.0 ml. yeast suspension was incubated at 37°C. for 30 min. The mixture was then centrifuged, and 1.0 ml. of the supernatant was pipetted into a flask followed by 8.0 ml. of zinc sulphate and 1.0 ml. of sodium hydroxide, as in the precipitation of serum proteins. After being allowed to stand for 30 min. the mixture was filtered and the inulin (total fructose) concentration was determined in the filtrate and in a similar, but unyeasted, solution

The recovery of inulin was 103% (Table 4) which suggests that none of the material present was capable of being hydrolysed by yeast under the conditions used. The yeast was able to ferment glucose and fructose under similar conditions.

This experiment was repeated using inulin solutions made by diluting 10% (w/v) solutions of inulin which had been prepared as for injection and kept for various times at 100°C. The resulting solutions were treated with yeast as in the previous experiment. Again yeasting made no difference to the apparent inulin concentration, the recoveries averaging 101.3% (97-103%). (Table 4). It was also found that heating the inulin solution for various

TABLE 4. Effect of Yeasting on Inulin which had received various heat treatments.

Determinations in duplicate.

Solution	Heat Treatment of Inulin Soln.	Inulin concentration before yeasting	Inulin concentration after yeasting	Recovery
		mg./100 ml.	mg./100 ml.	%
1.	Dissolved 80°C	19.8	20.4	103
2.	Dissolved 80°C	20.0	20.2	101
	Dissolved 80°C + 5 min. at 100°C	19.9	20.6	103
	Dissolved 80°C + 30 min. at 100°C	19.5	20.3	104
	Dissolved 80°C + 60 min. at 100°C	19.7	19.4	98.5
3.	Dissolved 80°C + 5 min. at 100°C	20.0	19.4	97
	Dissolved 80°C + 30 min. at 100°C	19.1	19.5	102
	Dissolved 80°C + 60 min. at 100°C	19.9	20.4	103

times made no difference to the apparent inulin concentration. This was to be expected since any decomposition of the inulin to smaller fragments would not alter the total fructose concentration.

Experiments on the reducing power of inulin solutions.

The reducing power of inulin is slight and is presumably due to the terminal fructose units.

In order to examine the effect of prolonged heating on the reducing power of inulin solutions, the Hagedorn and Jensen (1923) method for the determination of reducing substances was employed. The amount of reducing material was determined in inulin solutions containing 20 mg./100 ml. and prepared by diluting 10% (w/v) solutions of inulin in 0.9% (w/v) sodium chloride solution which had been kept at 100°C. for various times. This treatment caused the reducing capacity of the inulin solutions to be doubled (Table 5).

Since this treatment was more drastic than that received by an inulin solution being prepared for administration a second experiment was carried out in which the conditions were similar to those actually employed.

A 10% (w/v) solution of inulin was made by heating the inulin in 0.9% (w/v) sodium chloride solution to 100°C. for 60 sec. and this was then allowed to cool. After 20 min. the inulin solution was again brought to the boil for 30 sec. and again allowed to cool. This procedure was repeated for a further two periods of 20 min. Aliquots were removed from the inulin solution after the initial boiling and after subsequent boilings and the reducing power of these solutions, diluted, was determined (Table 5). There was again an increase in the reducing power.

TABLE 5. The Effect of Heat Treatment on the Reducing Power of Inulin Solution

Heat Treatment	Reducing power as glucose mg./mg. Inulin
Dissolved 80°C	0.075
" " + 5 min. 100°C	0.073
" " + 30 min. 100°C	0.098
" " + 60 min. 100°C	0.122
Dissolved 80°C	0.045
" " + 1 min. 100°C) + cool 20 min. + 1 min. 100°C)	0.057
+ cool 20 min. + 1 min. 100° C	--
+ cool 20 min. + 1 min. 100° C	0.125

Conclusions

The observed increase in reducing power of inulin solutions produced by prolonged heating, without the production of any yeast-fermentable material suggests that the inulin molecules had undergone decomposition to fragments which were still too large to be metabolised by yeast under the conditions employed. The presence of such fragments in an inulin solution could cause, as has already been described, an error in the determination of inulin clearance and it must be admitted that in some of the clearance experiments carried out before this phenomenon was noticed, inulin which had been subjected to such treatment was used. Unfortunately it is not possible to estimate the error involved. In the above experiments the reducing power was never increased by more than 100%. An increase in reducing power of this magnitude could involve, in the extremes, either the hydrolysis of one molecule of fructose from each inulin molecule or the hydrolysis of each inulin molecule into two fragments of approximately equal size. In the first instance it would be fair to assume that the inulin molecule minus one fructose unit would still behave physiologically like inulin; in the second, the properties of the hypothetical fragments cannot be ascertained. Thus without knowledge of the nature of the decomposition which appears to take place on heating it is impossible to estimate what effect the use of such inulin solutions would have on the determination of the inulin clearance. The results of Josephson and Lindahl must, however, be borne in mind.

III. The Relationship between the Inulin Plasma Concentration and the Rate of Excretion.

Procedure

Five experiments in which the rate of inulin excretion was measured at three or four different plasma concentration levels were carried out on four male subjects. Of these subjects two were normal healthy individuals (W.H. and W.R.); the other two had mild diastolic hypertension but were otherwise perfectly normal.

The experiments were carried out while the subjects were in the post-absorptive state. To ensure a sufficient rate of urine flow about 500 ml. of water were given to the subjects to drink at the start of the experiment and during the course of the experiment water was given at the approximate rate of 200 ml. every 30 min., making a total of about 1500 ml.

The inulin used was that supplied by T. Kerfoot and Sons. It was prepared for use by dissolving it in sterile pyrogen-free 0.9% (w/v) sodium chloride solution at 80-100°C. and passing the solution through an autoclaved Seitz filter. The solutions were of concentrations of 5, 10 or 15% (w/v) and although they were supersaturated, they remained without precipitation for several hours. When the inulin had precipitated from the solution the latter was warmed to 80°C. to redissolve the inulin and cooled to 37°C. before injection.

Inulin clearances were measured at three or four different plasma concentration levels, with three or four clearance periods at each level. Plasma inulin concentrations were established and

maintained by appropriate priming and sustaining infusions given into a vein in the forearm. In the initial experiments the infusion was maintained by a gravity feed system, the rate being controlled by a long screw clamp attached to the rubber tubing from the reservoir. Plasma concentrations were maintained at different levels by altering the rate of infusion and the concentration of inulin in the infusate. In the last experiment a constant-spread infusion pump, delivering 0.60 ml./min. was employed and different plasma concentrations were obtained by altering the concentration of the infusate. The constant speed jump gave the more constant plasma concentrations. In the first four experiments creatinine was also infused at various rates to provide different plasma creatinine levels. In the last experiment creatinine was infused at a constant rate so that the plasma creatinine concentration remained approximately constant throughout.

Blood was obtained at the start of the experiment for estimation of the inulinoid blank, and at the beginning and end of each clearance period, by venepuncture using the arm opposite to that receiving the infusion.

Clearance measurements were started 30 min. after the start of the sustaining infusion and clearance periods were 10-20 min. in duration. Urine was collected by an indwelling urethral catheter and at the end of each collection period the bladder was washed out once with 20 ml. sterile 0.9% (w/v) sodium chloride solution, followed by insuflation and expression of air.

Inulin in serum and urine was estimated according to the method already described and duplicate analyses were carried out

whenever possible. Clearances were calculated by dividing the mean minute rate of excretion of inulin by the mean of the plasma concentrations at the start and end of the period. No correction for delay-time was considered necessary since the plasma concentrations were virtually constant at any one level. The clearances were not corrected to a standard surface area.

Results.

The appropriate details of each experiment are given in Tables 6-10. The combined results of these experiments have been analysed in two ways.

1. By comparing the values obtained for inulin clearances at different plasma levels.

2. By analysis of the relationship between the rate of excretion of inulin and the plasma concentration.

The results of inulin clearance determinations at different plasma levels are given in Table 11A. In order that different absolute clearances should be comparable the clearances have been expressed as a percentage of the clearance determined at a plasma level of approximately 100 mg./100 ml., since this level is common to all experiments. For comparison the relevant data obtained by Ferguson et al. is given in Table 11B.

It will be seen that the mean percentage clearances differ slightly from 100% but in no case is this statistically significant.

Regression analysis of data requires consideration of the experimental errors involved, which are of two types.

1. Errors attributable to variable emptying of the bladder and timing of the collections.

2. Errors in the chemical estimation of inulin in plasma and urine.

The errors of the second type may for the present purpose be considered to be negligibly small compared to those of the first type, (standard deviation of chemical analysis of serum = 1.7%; of urine 1.4%). Thus the error in determining P will be small compared to that in determining UV.

In view of this the linear regression line, relating the experimentally determined values of UV to P were calculated. The magnitude and algebraic sign of the intercept of these lines on the UV-axis, and the error of the estimate of the intercept were then calculated so that the significance of the difference between the intercept and zero could be determined. (For method employed see appendix).

A summary of the statistics for the group of experiments is given in Table 12A. From this it can be seen that in no case is the intercept different from zero by a significant amount. (p less than 0.05), although it approaches significance in the case of W.P., where p is less than 0.1. The intercept is positive in three cases - the same three cases in which the clearance decreased with increasing plasma inulin concentrations. This follows since, when the ratio $\frac{UV}{P}$ decreases while P is increasing, the lines connecting the experimental points to zero must have slopes which decrease with increasing P, so that the best fitting line will have a positive intercept on the UV-axis. The converse is true when the clearance of inulin increases with increasing plasma concentration. For comparison the linear regression lines fitted to the infusion experiment data of Ferguson et al. are given in Table 13.

TABLE 6. Inulin Infusion Expt. 1. Subject W.R.

Time	Procedure	Period Duration	Urine flow	Plasma Inulin Conc. (Mean)	Inulin excreted	Inulin Clearance	
min.		min.	ml./min.	mg./100 ml	mg./min.	ml./min.	
0	P.* 0.6 g. Inulin						
	S.* 5.0 mg./min. in						
38-50	2.0 ml. Saline	12.3	2.0	5.0 ‡	6.5	128	
-63		12.5	3.5	4.7	6.2	132	
-75		15.5	7.6	5.2	6.0	114	
-84		9.0	13.4	5.3	6.2	109	
-93		9.0	16.8	5.0	6.8	134	125
94	P. 1.8 g. Inulin						
	S. 30 mg./min. in						
123-133	1.0 ml. Saline	9.8	10.8	18.0	22.0	121	
-147		13.5	8.6	21.2	22.5	106	
-159		11.8	8.2	22.4	26.1	114	
-170		11.3	6.2	21.1	22.2	105	112
172	P. 3.0 g. Inulin						
	S. 75 mg./min. in						
210-222	2.5 ml. Saline	12.3	5.0	61.2	73.5	120	
-235		13.0	4.5	58.7	71.1	121	
-248		13.0	4.0	56.2	63.7	113	
-260		12.0	3.3	55.2	61.5	112	117
265	P. 6.0 g. Inulin						
	S. 150 mg./min. in						
290-302	5.0 ml. Saline	11.5	8.9	123.1	166	137	
-313		11.0	10.0	118.2	155	132	
-315		11.5	10.3	114.2	159	138	
-321		6.0	8.8	112.2	134	120	132

‡ At this level the plasma concentration being not greatly different from the inulinoid blank was not considered to be sufficiently reliable and therefore the clearance determinations at this level have not been included in subsequent analyses.

* P. - priming injection.
S. - sustaining injection.

TABLE 7. Inulin Infusion Expt. 2. Subject J.C.

Time	Procedure	Period Duration	Urine flow	Plasma Inulin Conc. (Mean)	Inulin excreted	Inulin Clearance	
min.		min.	ml./min.	mg./100 ml.	mg./min.	ml./min.	
0	P. 0.6 g. Inulin S. 5.0 mg./min. In 2.0 ml. Saline						
30-47		17.0	3.5	12.0	12.8	107	
-60		13.0	5.0	9.8	10.6	108	
-73		13.0	6.4	9.1	9.9	109	
-86		12.5	5.5	8.4	9.2	109	108
86	P. 1.8 g. Inulin S. 30 mg./min. in 1.0 ml. Saline						
96-106		9.5	10.1	28.7	34.1	119	
-120		14.5	4.1	31.1	32.4	105	
-138		18.0	4.3	34.2	34.6	101	
-150		12.5	7.2	36.2	43.1	119	111
152	P. 3.0 g. Inulin S. 75 mg./min. in 2.5 ml. Saline						
192-202		10.0	9.5	77.5	82.1	106	
-216		14.0	4.7	66.0	72.6	110	
-218		11.5	3.6	61.7	65.9	107	
-231		14.0	3.4	61.7	63.5	103	107
235	P. 6.0 g. Inulin S. 150 mg./min. in 5.0 ml. Saline						
269-280		11.0	2.8	171	193	113	
-289		9.0	12.9	162	210	129	
-296		6.5	14.7	136	245	157	
-306		10.0	12.4	176	211	120	130

TABLE 8 Inulin Infusion Expt. 3. Subject C.M.

Time		Procedure	Period Duration	Urine flow	Plasma Inulin Conc. (Mean)	Inulin Excreted	Inulin Clearance	
min.			min.	ml./min.	mg./100ml	mg./min.	ml./min.	
0	P.	3.0 g. Inulin						
	S.	20 mg./min. in 1.0 ml. Saline						
64-74			13.3	3.7	33.5	24.0	72	
-86			12.5	4.4	32.1	26.3	82	
-97			11.0	10.7	31.4	25.4	81	
-106			9.0	14.7	30.4	27.9	92	82
115	P.	4.5 g. Inulin						
	S.	50 mg./kin. in 2.5 ml. Saline						
154-168			14.3	6.0	65.4	52.4	80	
-181			12.8	4.4	61.4	45.4	76	
-195			14.0	3.1	59.6	39.6	67	
-222			26.5	3.3	57.5	43.2	75	74
224	P.	5.5 g. Inulin						
	S.	100 mg./min. in 5.0 ml. Saline						
244-258			14.0	2.3	121	89.5	74	
-274			16.0	1.8	118	74.5	(64)*	
-290			15.5	1.6	115	87.0	76	
-366			16.0	1.4	108	66.4	(61)*	75

* Incomplete collection - leakage at catheter.

TABLE 9. Inulin Infusion Expt. 4. Subject W.H.

Time	Procedure	Period Duration	Urine flow	Plasma Inulin Conc. (Mean)	Inulin excreted	Inulin Clearance	
min.		min.	ml./min.	mg./100 ml.	mg./min.	ml./min.	
0	P. 2.5 g. Inulin S. 25 mg./min. in 1.0 ml. Saline						
41-55		14.5	2.0	30.3	28.8	95	
-67		12.3	3.1	28.4	27.5	96	
-38		11.0	3.7	27.6	28.6	103	
-89		10.8	3.7	25.3	25.7	101	99
89	P. 2.5 g. Inulin S. 50 mg./min. in						
126-138	2.0 ml. Saline	11.5	7.3	52.8	55.0	102	
-153		15.3	8.6	54.1	54.1	101	
-164		10.8	7.7	54.1	50.2	93	
-176		12.8	9.4	54.0	53.7	100	99
177	P. 2.5 g. Inulin S. 75 mg./min. in						
211-220	3.0 ml. Saline	8.5	16.7	80.8	77.3	96	
-228		8.8	16.0	83.0	78.9	95	
-241		12.8	14.2	83.5	76.7	92	
-250		9.5	15.1	84.5	83.3	99	96
251	P. 3.5 g. Inulin S. 100 mg./min. in						
278-300	4.0 ml. Saline	22.0	14.0	138.7	123	89	
-309		9.0	11.9	143.0	143	100	
-318		9.3	8.0	141.9	109	(77)*	
-329		11.0	9.9	138.2	125	91	93

* Incomplete collection - leakage at catheter.

TABLE 10. Inulin Infusion Expt. 5. Subject W.H.

Time		Procedure	Period Duration	Urine Flow	Plasma Inulin Conc. (Mean)	Inulin Excreted	Inulin Clearance		Creatinine Clearance	CCr / Cin.	
min.			min.	ml./min.	mg./100 ml	mg./min.	ml./min.		ml./min		
0	P.	0.5 g. Inulin							($P_{cr.}$ = 4·1 - 4·7)		
	S.	16 mg./min. in									
29-41		0.6 ml. Saline	12	12.8	11.0	12.5	114		150	1.31	
-50			9.5	16.2	11.3	13.0	115		154	1.34	
-61			10	17.2	11.6	10.8	97.5	109	139	1.42	1.35
64	P.	4.0 g. Inulin							($P_{cr.}$ = 6·2 - 6·8)		
	S.	48 mg./min. in									
90-102		0.6 ml. Saline	12	12.1	53.3	47.9	96		120	1.25	
-117			14.5	10.0	49.7	43.6	92		123	1.34	
-133			18.5	5.9	49.7	35.9	83	90	120	1.45	1.35
137	P.	6.0 g. Inulin							($P_{cr.}$ = 8·3 - 8·6)		
	S.	140 mg./min. in									
169-184		1.1 ml. Saline *	14.3	11.7	117.0	106.5	91		124	1.37	
-198			14	13.6	125.0	98.1	79		127	1.61	
-211			13.5	8.0	129.0	92.0	92	81	114	1.58	1.52

*Two infusion pumps used.

TABLE 11 A. Summary of TABLES 3 - 7. Inulin Clearances at Different Plasma Levels.

Plasma level	5 - 10 mg/100ml.		25 mg/100ml.		50 mg/100ml.		100 mg/100ml.	
Subject	Cin.	% Cin. $\frac{P}{\cdot}$ 100	Cin.	% Cin. $\frac{P}{\cdot}$ 100	Cin.	% Cin. $\frac{P}{\cdot}$ 100	Cin.	% Cin. $\frac{P}{\cdot}$ 100
	ml/min.	%	ml/min.	%	ml/min.	%	ml/min.	%
W.R.	125	95	112	85	117	89	132	100
J.C.	108	83	110	85	107	82	130	100
C.M.	-	-	82	109	74	99	75	100
$W.H._1$	-	-	99	105	99	105	94	100
$W.H._2$	109	135	-	-	90	111	81	100
Mean	-	104%	-	97%	-	97%	-	100%

TABLE 11 B. Data of Ferguson et al. Inulin Clearance at Different Plasma Levels.

	C_{in} P_{5-10}	C_{in} - % of C_{in} $P_{\div 100}$	C_{in} P_{25-10}	C_{in} - % of C_{in} $P_{\div 100}$	C_{in} P_{50}	C_{in} - % of C_{in} $P_{\div 100}$	C_{in} P_{100}	C_{in} - % of C_{in} $P_{\div 100}$
	ml./min.	%	ml./min.	%	ml./min.	%	ml./min.	%
C.M.$_1$	-	-	-	-	93	88	105	100
C.M.$_2$	83	74	-	-	98	87	112	100
W.R.	-	-	140	90	136	87	156	100
J.C.	-	-	88	94	-	-	94	100
Mean	-	74%	-	92%	-	87%	-	100%

Analysis of Combined Data.

Combined Means.	-	98%	-	95%	-	87%	-	100%

TABLE 12 A. Excretion of Inulin. Linear Regression Lines Fitted to Experimental Data. $UV = bP + a$.

Subject	Regression Equation	Variance of Estimated Intercept	t	n-2	p
W.R.	UV = 1.36 P - 7.25	12.9	2.0	10	0.1
J.C.	UV = 1.30 P - 6.44	36.6	1.1	14	0.4
C.M.	UV = 0.73 P + 1.68	14.0	0.5	8	0.7
$W.H._1$	UV = 0.91 P + 3.34	4.2	1.6	13	0.2
$W.H._2$	UV = 0.77 P + 4.33	13.9	1.2	7	0.2

To be significant p must be less than 0.05.

TABLE 12 B. Curvilinear Regression Lines. $UV = aP^b$.

Subject	Estimated b	Variance of b	t	n-2	p
W.R.	0.955	0.0037	0.74	10	0.2
J.C.	1.052	0.00068	2.0	14	0.1
C.M.	0.916	0.0038	1.4	8	0.2
$W.H._1$	0.951	0.00028	3.0	13	0.01
$W.H._2$	0.868	0.018	1.0	7	0.4

To be significant p must be less than 0.05.

TABLE 13. Excretion of Inulin. Linear Regression Lines Fitted to Inulin Infusion Data of Ferguson et al., 1950.

Subject	Regression Equation	Variance of Estimated Intercept	t	n-2	p
$C.M._1$	UV = 1.16p-5.7	14.1	1.52	7	0.2
$C.M._2$	UV = 1.09p-7.3	49.5	1.04	7	0.3
W.R.	UV = 1.70p-12.5	62.7	1.58	6	0.2
J.C.	UV = 0.97p-2.8	35.2	0.47	6	0.7

The above method of analysis assumes that the absolute error in determining either variate is independent of the value of the variate. Since all the chemical analysis are performed on final solutions of approximately the same concentration, obtained by appropriate dilutions of the serum filtrates or urine, the relative error of the determination is approximately constant. This means that the absolute error is proportional to the original concentration. This error applies equally to the terms UV and P since both contain the result of a chemical determination. The error in determining V is an inverse function of the absolute value of V. This follows from the fact that with small rates of urine flow the amount of urine which is unavoidably left in the dead space of the urinary tract, particularly in the bladder, may form an appreciable part of the total volume of urine secreted in a collection period; with large rates of urine flow the amount left in the dead space is negligible compared to the total amount excreted. In most of the periods the volumes of urine excreted was sufficiently large to make the error in determining V approximately constant and for this reason it was not considered necessary to allow for a changing relative error in the determination of V.

The absolute error in the variates UV and P is not constant but varies with the absolute values of the variates - in these experiments over a 15-fold range. Thus, the error, which is absolute, in the estimation of the intercept is so exaggerated by the large absolute variations of the points away from the zero co-ordinates that a significant departure of the intercept from zero is very difficult to detect.

In an attempt to overcome this difficulty a second method of analysis was performed. In this it is assumed that UV is proportional to P^b; if in fact UV is directly proportional to P the term b must be unity. If the logarithms of the experimentally determined points are plotted against one another the slope of the straight line which best fits the new points (log UV and log P) equals b. Thus:-

$$\text{If} \quad UV = aP^b$$

$$\log UV = b.\log P + a$$

(Note that a = clearance, $\frac{UV}{P}$, when P = 1.0 mg./100 ml.)

The slope of the logarithmic regression line can be calculated together with the error of the estimate, and from this the significance of the difference between the estimated value for the slope, b, and unity. Thus it is possible to determine whether or not the relationship between UV and P is one of direct proportionality - the hypothesis to be tested.

A summary of the statistics calculated in this way for the five experiments is given in Table 12B. From this it will be seen that the slope of the logarithmic line is significantly different from unity in only one of the five experiments. In this experiment b has been calculated to be less than unity so that the line best fitting the experimental points is a curve which is convex upwards, (i.e. consistent with the tubular excretion of inulin). It should be noted that in the original data from this experiment the change of mean inulin clearance with plasma concentration is not marked. However the good agreement between clearance values obtained for each level is responsible for this result. In the

other experiments UV is directly proportional to P within the experimental error.

The general conclusion which can be drawn from these analyses of the data is that the rate of excretion of inulin is proportional to the plasma concentration. However a final discussion of the results is postponed until after a consideration of delay time effects.

The Effect of Delay-Time on the Determination of Renal Clearances.

As has been pointed out by Smith (1951) there are two main difficulties in the determination of the renal clearance of a substance when the plasma concentration of this substance is changing. One is due to the arterio-venous concentration difference - a difficulty which can be overcome by the use of arterial blood samples; the other is due to the time delay which is inherent in the renal excretion mechanism.

When a substance which is excreted by the kidney is injected into a forearm vein a short interval elapses before the substance appears in the bladder urine. This interval is termed the first appearance time or minimal appearance time. It is the sum of:-

1. The circulation time from the site of injection to the glomerular or tubular vessels.
2. The time taken to cross the glomerular or tubular barrier.
3. The time required to pass along the tubules, collecting ducts and ureters into the bladder.

The total volume of the lumens of the tubules, collecting ducts and ureters is termed the "dead space". The time required

to pass through this dead space might be expected to vary inversely with the rate of urine flow, whether the flow of urine was laminar or with a solid front, as long as the dead space remained of constant volume. Bojesen (1949), however, has reported that the volume of the renal pelvis varies directly with the urine flow; Brodie and Mackenzie (1914) have reported from direct observation that the tubules dilate during diuresis; and Morales, Crowder, Fishmann and Gomers (1950) have deduced functional evidence that the volume of the dead space varies directly with the rate of urine flow. These changes tend to make the first appearance time less dependent on the rate of urine flow than it would otherwise be.

Because of the different lengths of the pathways travelled by different molecules there is a spread in the times taken by different molecules of the injected substance to reach the bladder. The first appearance time represents the time taken by the molecules travelling the shortest route. The time taken in travelling the longest route is much longer than this. In addition since it is considered likely that the flow of urine in the tubules is laminar and not with a solid front, the time taken to displace completely the "old" urine in the dead space with "new" urine containing the injected substance is also longer than the first appearance time. For these two reasons it is to be expected that the equilibrium time, i.e. the time required for urine to come into equilibrium with plasma with regard to a substance of which the concentration in plasma has been changed is longer than the first appearance time.

Smith, Goldring and Chasis (1938) measured the first appearance

time in man by injecting phenol red into the forearm vein and noting the time which elapsed before the phenol red appeared in the urine. At urine flows from 2 - 6 ml./min. this interval averaged 2.5 min.; at urine flows of 20 ml./min. it averaged 2.0 min.; and at urine flows of 1 ml./min. it averaged 3.3 min. There was, therefore, a slight inverse proportionality between the rate of urine flow and the first appearance time.

As a correction for this delay in the calculation of clearances from data obtained while the plasma concentration was changing these workers and many others subsequently, deducted 2.5 min. from the nominal mid-point of the collection periods to obtain appropriate plasma concentration.

Morales et al. (1950) measured, in dogs, the first appearance time, of substances excreted by glomerular filtration only as well as that of substances which are excreted by the tubules. They found that the first appearance time at very low rates of urine flow averaged 130 sec. decreasing to 80 sec. as the rate of urine flow was increased to 0.5 ml./min. and then remaining constant until the urine flow reached 2.0 ml./min. As the urine flow further increased the first appearance time increased to 100 sec. and then, as the urine flow became maximal, decreased to 70 sec. There was observed, thus, a varying relation of the first appearance time to the rate of urine flow though the overall decrease from the lowest urine flow to the highest was relatively small. These authors noted no difference in the pattern followed by substances which were excreted by glomerular filtration only from that followed by substances which were, in addition, excreted by the tubules.

In this same paper one of the authors, Gomez, put forward a mathematical expression for the calculation of the equilibrium time from the first appearance time based on the assumption that the flow of urine in the tubules is laminar.

The expression is:- $$Te = \frac{Ta}{\sqrt{1-s}}$$

where Ta is the first appearance time, Te is the equilibrium time and s is the fractional attainment of equilibrium. From this it will be seen that with a first appearance time of 2.5 min. the attainment of 99% equilibrium would require 25 min.

Chinard (1952) investigated one aspect of delay time by studying the excretion of several substances injected simultaneously into the renal artery of dogs. Urine was collected by ureteric catheter at intervals of 12 sec., and the amounts of each substance excreted in each period were measured. The waveform of the concentrations of the injected substance in the blood reaching the kidney was assumed to be approximately square and correction for recirculation was made by collecting urine similarly from the other kidney.

In one experiment creatinine, inulin and p-amino hippuric acid, (PAH), were injected. Creatinine* and PAH appeared at 96 sec. after the injection and inulin at 108 sec. All three reached maximum excretion at 120 sec. The fraction of PAH excreted by the tubules (measured as the total amount of PAH excreted less the amount filtered as estimated by the amount of inulin excreted) did not reach maximum rate until 156 sec. after the injection. After reaching maximal rate the excretion of each substance gradually decreased to zero but did not reach this value until after 5 min.

* Allowance was made for the excretion of endogenous creatinine.

Three conclusions may be drawn from this data.

1. After an almost instantaneous injection into the renal artery the rate of excretion of these three substances does not reach a maximal value until a short interval after they first appear and does not approach zero until some minutes later. The equilibrium time is thus considerably longer than the first appearance time in agreement with the conclusions of Gomez.

2. The maximal rate of tubular excretion is not reached until after the maximal rate of glomerular excretion. The author considered this an expression of the time required to pass from the peritubular capillaries, across the tubular wall into the lumen of the tubules.

3. The appearance time of inulin measured in this way is longer than that of creatinine.

The conclusion that the equilibrium time is longer than the first appearance time was also reached by Brun et al. (1950) from consideration of anomalous diodone clearances obtained when the plasma concentration of diodone was rapidly decreasing or increasing. After correction for arterio-venous differences in plasma concentration they calculated that they must allow for a delay time of 5-8 min. in order to obtain clearances equal to those obtained with a constant plasma concentration. However, the very rapid changes in plasma concentration which occur after the injection of diodone make quantitative estimation of delay time in this way difficult.

In an attempt to measure more directly the effective delay-time Michie and Michie (1951) studied the excretion of various

substances after the start of a continuous infusion immediately preceded by a priming injection. A constant plasma concentration was reached in about 10 min. whereas the rate of excretion did not reach a constant value until some 20-30 min. later. Interpretation of this finding is made difficult, however, by the high initial plasma concentration produced by the priming injection, but nevertheless it would appear from this data that the equilibrium time is in the region of 25 min., i.e. some ten times longer than the first appearance time, again in agreement with the conclusions of Gomez.

A somewhat different approach to the problem of delay time has been made by several groups of investigators studying the excretion of radio-active substances, although it was not always appreciated that this was, most probably, the phenomenon which was being studied.

Govaerts (1947a, b) noted that after a single intravenous injection of inorganic phosphate containing radio phosphorus (P^{32}) in dogs the specific activity of the phosphate in the urine was 30% higher than that of the plasma inorganic phosphate 10 min. after the injection. Thereafter the specific activities of the plasma and urinary inorganic phosphate approached one another becoming approximately equal after 60-90 min. Govaerts concluded that the inorganic phosphate in plasma was largely in a non-diffusible form and that the inorganic phosphate normally excreted was derived from a small diffusible fraction.

Handler and Cohn (1951), though they were able to confirm Govaerts' findings, were not able to demonstrate the presence of

non-diffusible inorganic phosphate in plasma using radio-active phosphate in dialysis experiments. Using injections which resulted in increasing plasma inorganic phosphate specific activities they obtained no disparity between the plasma and urinary specific activities and they concluded that the effect noted by Govaerts was due in some way to a delay-time effect.

Bradley, Nickel and Leifer, (1952) quoted unpublished data of Rittenberg and San Pieta who found that after an intravenous injection of urea containing N^{15} the specific activity of the urea in the urine exceeded that of the plasma for more than 60 min.

Bradley et al. noted a similar effect with radio-sodium (Na^{24}) and with radio-potassium (K^{42}), the effect being greater with the latter. Differences in the urinary and plasma specific activities as great as 700% were sometimes observed and the effect was independent of the urine flow. The authors concluded in agreement with Handler and Cohn that the phenomenon was due to a delay-time effect.

Radio-sodium leaving the plasma takes an appreciable time to reach the bladder. Since the specific activity of the plasma decreases immediately after the injection as a result of the distribution of the radio-sodium throughout the sodium space of the body, the specific activity of the sodium in the plasma at the time of urine sampling is less than at the time when the excreted sodium left the plasma. As the rate of decrease of plasma sodium specific activity becomes less, i.e. as uniform distribution throughout the sodium space is approached, the plasma and urinary specific activities tend to become equal.

A similar reasoning can be applied to the excretion of inulin. The rate of excretion of inulin (UV) divided by the glomerular filtration rate (F) gives an expression (UV/F) which represents the theoretical concentration of inulin which would exist in urine if inulin was excreted solely by filtration and if there was no reabsorption or excretion of water by the tubules. The plasma inulin concentration (P) is thus analogous to the plasma specific activity and UV/F to the urinary specific activity.

Bradley et al. investigated the excretion of inulin following a single intravenous injection. By assuming that the glomerular filtration rate (F) was constant and equal to the equilibrium inulin clearance they were able to relate UV/F to P (actually they related UV to PF) and they obtained a relationship qualitatively similar to that obtained for plasma and urinary specific activities of radio-sodium or radio-potassium. They concluded that a linear relationship exists between UV and P for inulin but that after a single injection this is distorted by the delay-time effect so that inulin clearances following a single injection show an apparent dependence on the plasma concentration.

It might at first seem possible to test this hypothesis by comparing the disparity between the plasma and urinary specific activities with the disparity between plasma inulin concentration and the parameter UV/F determined simultaneously. However, it is clear that the disparity is due to two factors:- 1. the rate of decrease of the plasma component (specific activity or inulin concentration); and 2. the delay-time. Because of the different diffusion coefficients of sodium and inulin the rates of decrease of the plasma specific activity and plasma inulin concentration,

respectively, are different so that without knowing the exact relationship of plasma specific activity and of inulin plasma concentration to time the delay effect cannot be compared for these substances.

It is possible, however, to compare the disparities indirectly. Bradley et al. constructed from their data what they termed "cumulative frequency distribution curves" of delay time. These were taken to represent the fraction of total active nephrons producing a delay-time of less than a certain value. From these curves it was concluded that on the average 75% of the glomerular filtrate is delayed by less than 10 min. 20% by between 10 and 20 min. and the rest by an even longer period. In a few experiments the values for the distribution of delay were obtained simultaneously for radio sodium, radio potassium and inulin. In spite of differences in the relative disparities observed, there was general agreement between the delay-time distributions observed for these substances in any one experiment and the authors concluded from this that the plasma-urine disparities were due entirely to the delay-time effect.

Realising the impossibility of testing directly the hypothesis put forward by Bradley et al., viz. that the non-proportionality between the rate of excretion and the plasma concentration after single injections of inulin is due to delay-time effects, it was considered, nevertheless, that the examination of the excretion rates of simultaneously injected radio sodium, inulin and creatinine might give useful information regarding the mechanisms of excretion of these substances and accordingly two experiments were carried out in which this was done.

Experimental.

Two experiments were carried out on the same subject at an interval of two weeks. The subject was in the post-absorptive state and in the first experiment was encouraged to drink as much water as possible in order that a high rate of urine flow should be obtained. In the second experiment the subject was given only a small amount of water to drink in order that a sufficient, but not high rate of urine flow should be obtained. Otherwise the experiments were carried out as identically as possible.

A single injection of inulin 10 g., creatinine 10 g. and radio sodium in 100 ml. 0.9% (w/v) sterile sodium chloride solution was given as quickly as possible. Samples of venous and arterial blood were obtained from the contralateral arm at times from 3-150 min, after the injection. Urine was collected by means of an indwelling urethral catheter at appropriate intervals, corresponding roughly with blood sampling.

Inulin was determined in serum and urine by the method previously described; creatinine was determined by the method to be described in the section on creatinine estimation; sodium was determined chemically using a Barclay Internal standard flame photometer; and radio activity was determined by by means of an E.K. Cole Scaler.

Results.

These are given in Tables 14 and 15. Inulin and creatinine clearances were calculated using the mean rate of excretion and the mid-period plasma concentrations obtained from a smoothed curve drawn through the experimentally determined points. In

TABLE 14. Delay time Expt. 1.

Period	Urine flow	Time after injection	Mid Period Time after injection	Inulin			Creatinine			Ccr/Cin
				P (mid-pt.)	UV	Cin	P (mid-pt.)	UV	Ccr	
	ml./min.	min.	min.	mg./100ml	mg./min	ml./min.	mg./100 ml	mg./min	ml/min.	
1.	5.8	-	-	-	-	-	0.85	1.13	135	1.50
2.	15.7	3	1.5	-	-	-	-	-	-	-
3.	21.8	7.3	5.1	14.5	165	114	54	93.5	173	1.52
4.	19.2	13.3	10.3	110	120	109	39	61.5	158	1.45
5.	20.8	22.3	18.0	85	79.5	93.5	31.5	40.5	128	1.37
6.	19.8	35.3	29	63	51.5	82	26	31.2	120	1.47
7.	18.0	47.5	41	47.5	40	84	21.5	27.2	110	1.71
8.	19.5	77.5	62.5	32.5	27	83	17	19.8	117	1.41
9.	6.5	99.5	88.5	23.0	21	96	14	16.0	114	1.19
10.	3.1	119	109	19.0	16.5	87	12.7	13.5	106	1.22
11.	3.0	136	124	14.5	13.7	94	11.5	13.1	114	1.21

TABLE 14 (Contd)

Period	Mid-period time after injection	Inulin		Creatinine		Radio-sodium		
		$\frac{UV}{P \times 90}$ *	$\frac{UV}{90} \times 100$	$\frac{UV}{P \times 113}$ ‡	$\frac{UV}{113} \times 100$	S.A. plasma	S.A. urine	$\frac{S.A.u}{S.A.p}$
1.								
2.	1.5							
3.	5.1	1.28	183	1.55	95	32	72	2.25
4.	10.3	1.21	134	1.41	57	31.5	45	1.43
5.	18.0	1.04	95	1.15	30.4	29	30	1.23
6.	29	0.91	57	1.07	27.2	26	32	1.23
7.	41	0.94	44.5	0.98	20	24	27	1.13
8.	62.5	0.92	30.5	1.05	17.5	22	24	1.09
9.	88.5	1.07	23.4	1.02	14	21.5	25.5	1.23
10.	109	0.97	18.3	0.95	12	21	16	0.76
11.	124	1.05	15.2	1.02	8	20	24.5	1.22

* A value of 90 was chosen since this was the mean inulin clearance of the periods 5 - 11.

‡ A value of 113 was chosen since this was the mean creatinine clearance of the periods 7 - 11.

TABLE 15. Delay Time Expt. 2.

Period	Urine flow	Time after injection	Mid Period Time after injection	Inulin			Creatinine			Ccr/Cin
				P mid-pt.	UV	Cin	P mid-pt.	UV	Ccr	
	ml./min.	min.	min.	mg./100ml	mg./min	ml./min.	mg./100ml	mg./min	ml./min	
1.	2.6	-	-	-	-	-	0.85	-	-	-
2.	6.5	6.3	3.0	-	-	-	-	-	-	-
3.	4.9	11.5	8.8	107	98	92	52	79	151	1.50
4.	7.3	20	15.8	65	79.5	122	35	65.6	187	1.53
5. & 6. *	4.5	48	34	37	39.5	107	16	26.5	158	1.48
7.	3.3	60	54	23	25.3	110	12.6	17.9	142	1.27
8.	2.8	86	73	19.5	21.4	110	11.7	16.3	139	1.26
9.	2.5	100	93	15.7	17.0	107	10.9	14.2	130	1.21
10.	2.0	121	111	13.3	12.3	93	10.2	10.6	103	1.11
11.	2.0	148	135	10.3	11.8	114	9.3	11.7	126	1.10

* Incomplete collection in period 5 with carry over into period 6.

TABLE 15 (Contd).

Period	Mid-period time after injection	Inulin		Creatinine		Radio-sodium		
		$\frac{UV}{P \times 108}$ ‡	$\frac{UV}{108} \times 100$ ‡	$\frac{UV}{P \times 115}$ ‡	$\frac{UV}{115} \times 100$ ‡	S.A. plasma	S.A. Urine	$\frac{S.A.u}{S.A.p}$
1.	-	-	-	-	-	-	-	-
2.	3.0	-	-	-	-	-	-	-
3.	8.8	0.93	91	1.31	73	132	168	1.27
4.	15.8	1.13	74	1.62	61	112	130	1.18
5. & 6. *	34	1.00	36.5	1.36	25	92	102	1.10
7.	54	1.02	23.4	1.23	16.6	86	101	1.10
8.	73	1.02	19.8	1.21	15.1	86	90	1.04
9.	93	0.99	15.7	1.13	13.2	86	87	1.01
10.	111	0.86	11.4	0.90	9.8	86	80	0.95
11.	135	1.15	10.9	1.10	10.9	86	77	0.90

* Incomplete collection in period 5 with carry over into period 6.

‡ See footnote, Table 14.

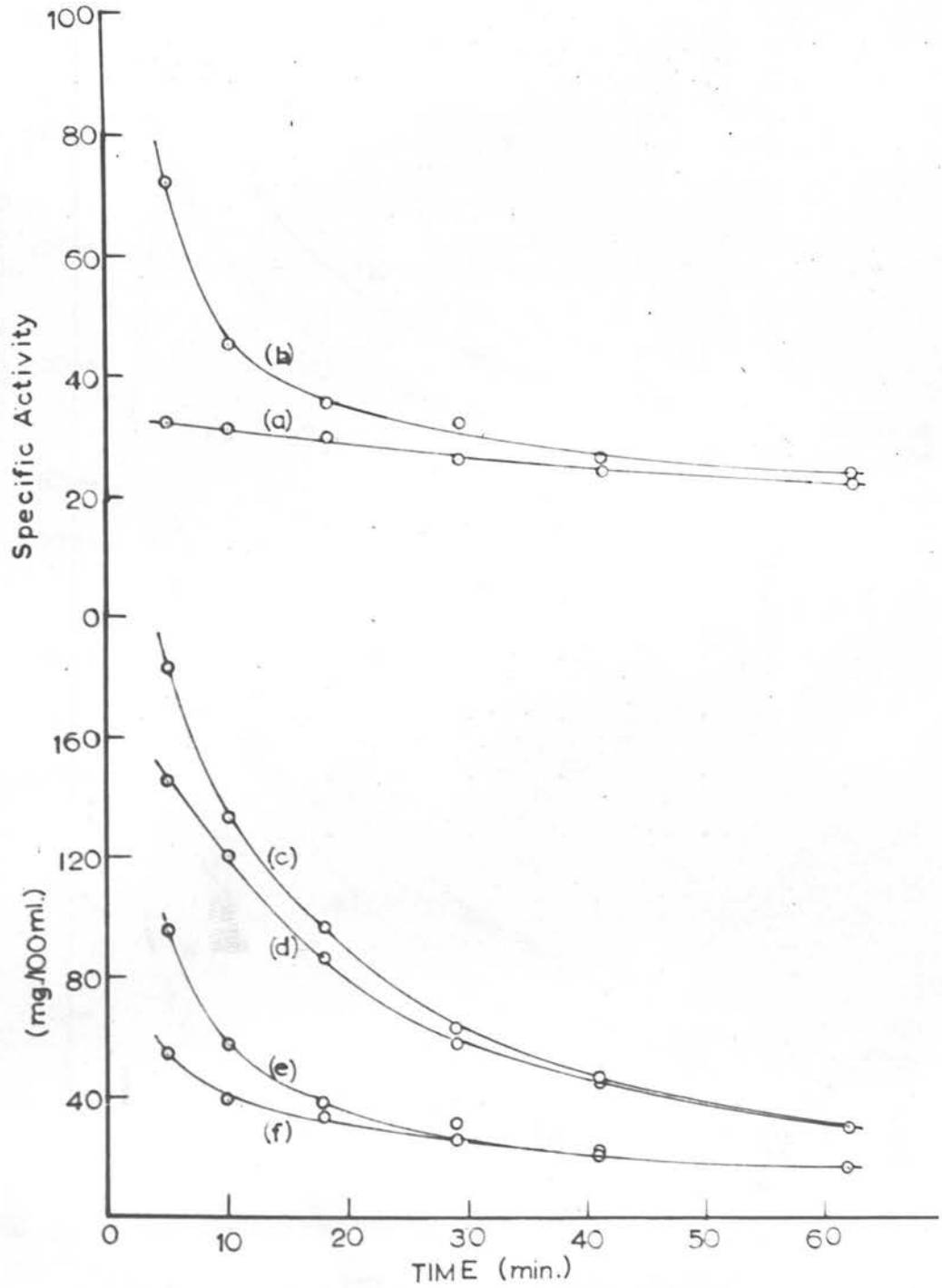

FIGURE 5. Delay-time Experiment No. 1.

(a) Specific activity of radio-sodium in serum; (b) specific activity of radio sodium in urine; (c) UV/F for inulin; (d) P for inulin; (e) UV/F for creatinine; (f) P for creatinine.

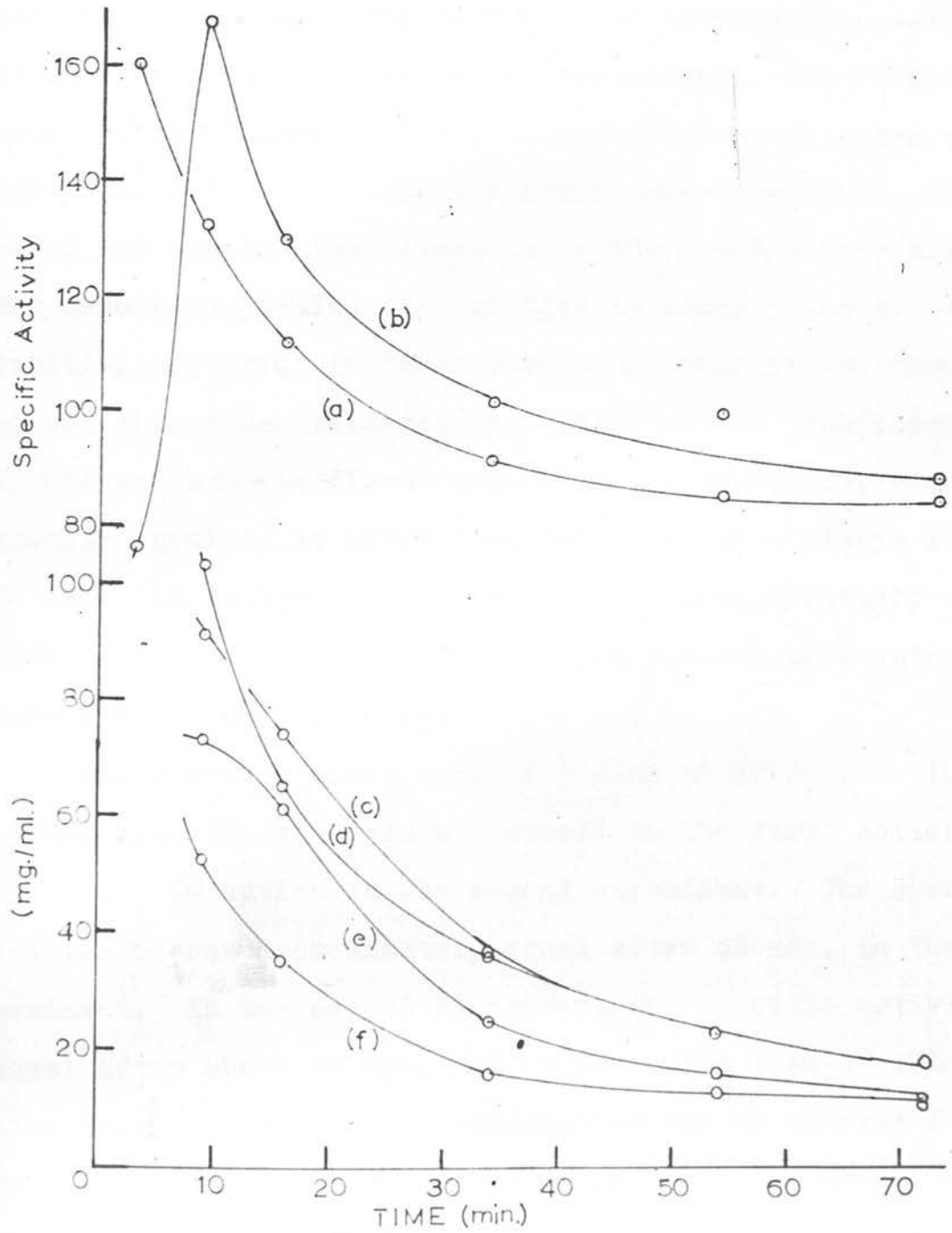

FIGURE 6. Delay-Time Experiment No. 2.

(a) Specific activity of radio sodium in serum; (b) specific activity of radio sodium in urine; (c) UV/F for inulin; (d) P for inulin; (e) UV/F for creatinine; (f) P for creatinine.

both experiments inulin and creatinine clearances were relatively high at first, reaching an approximately constant value after 30 and 40 min. respectively in the first experiment; and 50 and 100 min. respectively in the second experiment. The values for the ratio of the parameter, UV/F to the mid-period plasma concentration have been calculated both for inulin and creatinine, F in each case being the equilibrium clearance of these substances respectively, and have been plotted against time in Figs. 5 and 6. The disparities appearing in these curves and similar to, though less marked than these reported by Bradley et al. The parameter UV/F is higher than the plasma concentration initially, becoming approximately equal to it after a variable period - always longer than 30 min. It follows from this that there is initially a disparity between the rate of sodium excretion and the calculated filtered load for these substances.

The specific activities of sodium in urine were initially higher than those in plasma, except in the first collection period after the injection in the second experiment. The specific activities become approximately equal after 40 min. in the first experiment. In the second experiment the specific activities became equal after about 90 min. and subsequently that of urine was less than that of plasma. (No explanation can be offered for this. Possibly an experimental error). In general, these findings are similar to, though again less marked than those reported by Bradley et al.

The ratios of the filtered load to the rate of excretion for inulin and for creatinine, and of the urinary specific activity

to the plasma specific activity have been calculated and are also given in Tables 14 and 15. It will be noted that in both experiments all three ratios are initially greater than unity but gradually approach this value. The specific activities show the highest initial ratios. Inulin shows the lowest initial ratios especially in the second experiment where the initial ratios for inulin are scarcely different from unity.

Though it is not relevant to the present considerations it should be noted that in both experiments the creatinine/inulin clearance ratio progressively decreased. This is in agreement with the findings of Winkler and Parra (1937) and those of Shannon and Ranges (1941).

Conclusions.

These experiments show that after a single injection of inulin, creatinine and radio-sodium there is a disparity between calculated filter load and the rate of excretion of inulin and creatinine and also a qualitatively similar disparity between the specific activities of sodium in plasma and urine. Quantitatively the disparity is greatest for radio sodium and least for inulin. Thus the substance which showed the steepest initial fall in plasma concentration, i.e. radio-sodium, showed the greatest disparity, in agreement with previous conclusions. Inulin, which showed the least steep fall in plasma concentration, showed the least disparity.

It seems likely that the disparities observed were due to a delay-time effect, although the data by no means excludes other possibilities, e.g. non-proportionality between rate of excretion and plasma concentration due to tubular activity. One important

conclusion which is confirmed by this data is that the interpretation of clearance determinations while the plasma concentration is rapidly changing is fraught with difficulties. Theoretically equilibrium between plasma and urine will never be reached if the plasma concentration is changing. However, from a practical point of view it would seem from this data that the disparity between plasma and urine for inulin and creatinine is not great 60 min. after an injection, at least compared to the experimental error of clearance determinations. Thus part of the discrepancy already noted between the data of Ferguson et al. relating to the excretion of inulin after a single injection and that of Hogeman (1943) and Ahlborg (1947) may be due to the fact that the former started measuring clearances 30 min. or sooner, after the injection whereas the latter did not start until 60 mins. after the injection.

Conclusions regarding the Excretion of Inulin in Man.

The data derived from the present series of experiments would seem, on the following grounds, to justify the conclusion that in man inulin clearance is independent of the plasma concentration over a considerable range, within the error of the methods involved in its measurement.

1. No consistent change was observed in the clearance of inulin at different plasma levels when a continuous infusion was employed to maintain inulin plasma levels. (See Table 11A).

2. Using statistical analysis, (See Table 12A, B), it was impossible to demonstrate that the rate of excretion of inulin (UV) was not directly proportional to the plasma concentration (P)

except in the analysis of the curvilinear regression line relating UV and P in one experiment, (Expt. No. 4., in which it appeared that $UV = aP^b$, where b was significantly less than unity - a result indicating that the inulin clearance was decreasing with increasing P).

Admittedly the validity of both these forms of analysis is dependent on the absence of consistent changes in renal function during the examination of the relationship between the two variates. It is quite possible that an effect of plasma concentration on inulin clearance could be masked by coincidental changes in renal function. Indeed, such changes in renal function, due possibly to the infusion of saline (containing the inulin) (Crawford and Ludeman, 1951; Crawford and Gaudino, 1952), or to the ingestion of considerable amounts of water, (Ladd, 1951), may have been responsible for the increases in inulin clearance which were observed during the last few collection periods in two of the experiments (Nos. 1 and 2).

Allowance for possible changes in renal function can only be made by comparing inulin clearances of some other indifferent substance, e.g. creatinine maintained at a constant plasma level. This was done in one experiment (No. 5) and the results of this showed that there was a slight tendency for the creatinine/inulin clearance ratio to increase as the plasma inulin level was increased. However the creatinine plasma level was not entirely constant, increasing from 4 to 8 mg./100 ml. during the course of the experiments and, although reference to the data on the excretion of creatinine shows that on the average creatinine clearance was found to be independent of the plasma over this

range, there was, in fact, a considerable individual variation observed so that the changes in creatinine/inulin clearance ratio observed in this experiment may have been due to the change in the plasma creatinine concentration.

Examination of the relevant literature reveals that the above conclusion is in agreement with those of the majority of investigators who have examined the relationship between the rate of excretion of inulin and the plasma concentration. In particular this was the conclusion reached by Kennedy and Kleh (1953) who examined this relationship in a series of 14 normal persons at plasma inulin levels, maintained by constant infusion, from 5 to 175 mg./100 ml. In view of this, therefore, it is necessary to seek some explanation of the apparently contradictory findings presented by Ferguson et al.

Taking first the larger series of single injection experiments it seems likely from consideration of this effect of delay-time that clearance determinations, carried out while the plasma concentration is changing are liable to errors, the magnitude of which cannot easily be determined. It would seem, at least, that any attempted correction for the delay-time effect requires the allowance of an interval longer than the 2.5 to 6.0 min. which was accepted as the "effective" delay interval for many years. In this respect it should be noted that the curve relating plasma inulin concentration to time following a single injection (Robson et al., 1949) is of such a form that the allowance of a longer delay time in calculating clearances would reduce the apparent clearance at high plasma levels more than at low levels, so that

there would be less tendency for the inulin clearance to decrease with falling plasma levels.

Inulin clearance data relating to the subjects of the single injection series, No. 3 and No. 6, is given in Table 16. Using the original data from the experiment the inulin clearances have been calculated, applying various delay-time corrections to the mean plasma inulin concentration. It will be seen that as the delay-time correction is measured from 2.5 min. to 20 min., the apparent dependence of inulin clearance on plasma concentration disappears. Other experiments in the series show the same phenomenon.

It would seem, therefore, that a delay-time effect could entirely account for the finding of decreasing inulin clearance after a single injection.

Considering the continuous infusion experiments (Table 11B) it is less easy to offer a satisfactory explanation for their findings. Application of statistical analysis to the data reveals, certainly, that in none of the four experiments is it possible to demonstrate that UV is not directly proportional to P (Table 13) but, as has already been pointed out, this form of analysis involves certain assumptions which are not always justified and, further, that experimental variation makes it difficult to obtain conclusive evidence from regression analysis in individual experiments. Considering the actual clearance values it is to be noted that in one of the three subjects (W.R.) the mean clearance at a plasma level of 50 mg./100 ml., while lower than the mean clearance at a plasma level of 70 mg./100 ml.., is also lower than the mean

TABLE 16. Effect of Various Delay-time Corrections on Inulin Clearances following Single Injections of Inulin Data of Ferguson et al. partly unpub. (1950).

Time after injection (mid-period)	Inulin Clearance			
	Delay-time correction	2½ min.	6 min.	10 min.	20 min.
min.		ml./min.	ml./min.	ml./min.	ml./min.
Subject No. 3					
25		104	96	81	--
45		94	90	84.5	70
65		97	94	89	82
85		85	82	80	74
109		82	81	79	24
Subject No. 6					
30		101	93	85	--
50		86	82	77	65
70		82	79	76	67
90		78	76	73	67
110		75	73	70	65
129		73	71	69	64
149		76	75	73	68

clearance at a plasma level of 30 mg./100 ml. In the other two subjects there is, however, a consistent tendency for the clearance to be lower at low plasma levels and the only explanation which can be offered for these findings, other than that put forward by the authors, is that coincidental changes in renal function were responsible for the observed effect.

In conclusion, it must be emphasised that the failure to demonstrate a dependence of the excretion rate of inulin on the plasma concentration does not necessarily imply that there is no reabsorption or excretion of inulin by the renal tubules. It does, however, imply that any dependence of inulin clearance on plasma concentration which exists is, of such a magnitude that it cannot be detected by available methods and that its effect is, therefore, of no consequence on the determination of inulin clearance using existing techniques. It must also be emphasised that the conclusions reached here do not imply that inulin clearance is identical with the glomerular filtration rate since the existence of tubular reabsorption or excretion of inulin in amounts proportional to the plasma concentration or of partial impermeability of the glomerular membrane to inulin would not effect the direct proportionality between the rate of excretion and the plasma concentration, although either would make quite invalid the use of inulin clearance as a measure of glomerular filtration rate.

II. THE DETERMINATION OF CREATININE IN PLASMA OR SERUM AND IN URINE.

II. THE DETERMINATION OF CREATININE IN PLASMA OR SERUM AND IN URINE.

The measurement of the renal clearance of a substance requires the accurate determination of the concentrations of the substance in plasma, or serum, and in urine. The problem of the determination of creatinine in these fluids has attracted much attention and although certain advances have been made, a completely satisfactory solution is still awaited. Most of the methods which have been proposed have been based on the colour reaction which takes place when creatinine is added to sodium picrate in an alkaline medium (Jaffe, 1886). This reaction, however, is not specific for creatinine and, furthermore, it is generally held that there are present in plasma or serum, and to a smaller extent in urine, one or more unknown substances which give a colour with alkaline picrate similar to that given by creatinine.

Different analytical procedures for applying the Jaffe reaction to plasma, serum or urine have been found, on direct or indirect comparison, to give different results and such discrepancies emphasise the need for careful control of the many factors which influence the extinction coefficients of alkaline picrate and alkaline creatinine picrate, e.g. the concentration of the reagents, the temperature and time of colour development and the presence of interfering substances.

The effect of these factors and of the method of preparing protein-free filtrates of plasma or serum has not always been appreciated in evaluating the results of creatinine determinations. Further, the use of creatinine-destroying bacteria or of adsorption techniques, which have been claimed to provide methods specific for the determination of creatinine, introduce additional sources of error.

In the initial experiments of the investigation on the renal excretion of creatinine a method, which was essentially that of Miller and Dubos (1937a), was used for the determination of creatinine in serum and urine. This method involved the use of the NC-bacteria to obtain true creatinine concentrations. Simultaneously with these experiments an investigation was carried out to examine, in detail, the factors influencing the determination of creatinine in serum and urine and this showed that while the method of Miller and Dubos was not the most satisfactory, the error introduced by its use was not great. In order, therefore, that the whole series of physiological experiments should be comparable the method of creatinine determination was kept the same throughout.

The interpretation of creatinine clearance measurements requires, therefore, consideration of the method used for the determination of creatinine and of the possible errors involved. For this reason the results of the investigation on the factors influencing the determination of creatinine are reported before an account of the investigation on the excretion of creatinine

although much of the work was, in fact, carried out subsequently. In doing this it has been found convenient to group together the relevant literature, experimental findings and discussion of the effect of each factor or group of factors and although this requires that the experimental data be split up into many sections, it enables the influence of each factor to be more clearly defined.

Materials and Methods.

Reagents

Creatinine. Standard solutions were prepared daily by dilution of a stock solution containing 400 mg. creatinine in 100 ml. 0.1N HCl. The stock solution was kept at 0°C. and was made up at intervals of not longer than four weeks.

Picric Acid. An aqueous solution, saturated at room temperature (18-20°C.), was prepared from picric acid (A.R.) which had been recrystallised twice from water. This solution was made up at intervals of a few days and kept in dark bottles, satisfied the criteria of purity proposed by Folin and Doisy (1917).

Sodium Hydroxide. (A.R.) 2.5N.

Sodium Tungstate. (A.R.) A 10% (w/v) solution of Na_2WO_4, $2H_2O$.

Sulphuric Acid. (A.R.) 0.66N.

Phosphate Buffer. 1N., pH 7.0 (Green, 1933).

Oxalic Acid. (A.R.) Saturated aqueous solution.

Lloyd's reagent. Hartmann-Leddon Co., U.S.A.

NC-bacteria. A suspension of these bacteria was prepared according to the method of Miller, Allinson and Baker (1939). The bacteria were grown on a medium containing:- creatinine 5g., NaCl 5g., Na_2HPO_4, $12H_2O$ 5.2 g., KH_2PO_4 1.7 g. and 'Bacto-yeast extract' (Difco Laboratories) 200 mg. in 1,000 ml. of tap water. This medium, adjusted to pH 7.0, was sterilised by autoclaving

at 15 lb./sq. in. for 15 min. About 1 ml. of an actively growing culture was used to inoculate 100 ml. of medium. After incubation at 37°C. for 2-3 days the culture medium was centrifuges, the bacterial sediment washed twice with distilled water and then resuspended in about 4 ml. of distilled water. This suspension was stored at 0°C. and remained active for a few weeks.

The organism was obtained initially in the form of a dried culture which was used to inoculate a creatinine-broth medium. After growth had taken place, a creatinine-yeast extract medium was inoculated. It was found possible to prepare dried cultures which remained viable for at least nine months by evaporating, under reduced pressure, a small amount of an actively growing culture to dryness at room temperature and storing in vacuo over P_2O_5 at 0°C.

The plasma, serum and urine used in this investigation on methodology were obtained from normal persons. The plasma was heparinised. In addition a short series of determinations were carried out using serum of persons with various degrees of renal dysfunction.

A unicam S.P. 600 spectrophotometer was used to measure optical density.

Methods.

The Jaffe reaction. This was carried out, unless otherwise stated, according to the method of Folin and Wu (1919). To

4.0 ml. of the solution containing creatinine were added 2.0 ml. of alkaline picrate solution and the colour was allowed to develope for 20 min. in a water bath at 20±0.2°C. The alkaline picrate solution was made up immediately before use by adding 1 vol. of sodium hydroxide to 5 vol. of picric acid. The optical density of the developed colour was measured at a wavelength of 520 mu, using water as a reference optical density. For determinations in plasma or serum filtrates, or in diluted urines, two standard solutions were included with each batch and a standard curve was constructed from these. All results are given in terms of creatinine.

Enzymatic destruction of creatinine using the NC-bacteria.

The method used was that described by Miller and Dubos (1937a).

Adsorption of creatinine on Lloyd's reagent. The method used was that described by Haugen and Blegen (1953). Standard creatinine solutions were included with each batch and the temperature adjusted to 20°C. before measurement of the optical density.

Precipitation of plasma, or serum, proteins. Method I: To serum 2 vol. were added water 6 vol., sodium tungstate 1 vol. and sulphuric acid 1 vol. (Miller and Dubos, 1937a). This gave a filtrate which is termed "neutral" filtrate, equivalent to serum diluted 1 in 5.

Method II: To serum 2 vol. were added water 3 vol., sodium tungstate 1 vol. and sulphuric acid 2 vol. (Brod and Sirota,

1948). This gave a filtrate which is termed "acid" filtrate equivalent to serum diluted 1 in 4.

Observations on the Jaffe Reaction

Light absorption by alkaline creatinine picrate.

The absorption spectrum of alkaline creatinine picrate shows maximum absorption at wavelengths around 490 mμ, but owing to the high optical density of alkaline picrate, i.e. in the reagent blank, at wavelengths below 500 mμ, filters having maximum transmission at higher wavelengths have generally been used. When measured with absorptiometers employing filters or diffraction gratings which provide a relatively large band-width, the developed colour is reported not to obey Beer's Law (Bonsnes and Tausky, 1945; Hawk, 1947; Lauson, 1951; Schoch and Camora, 1952; Hervey, 1953) but when instruments providing light of a narrower band-width are employed the developed colour is reported to obey Beer's Law over the required concentration range. (Borsook, 1935; Borsook and Dubnoff, 1940; Hervey, 1953). It has also been reported, however, that even with monochromatic light Beer's Law is not obeyed at all wavelengths (Garner, 1952).

To investigate this point the optical densities of alkaline creatinine picrate were measured at wavelengths from 480 mμ to 520 mμ and plotted against the concentration of creatinine (Fig. 7). As the wavelength increased the relationship between the optical density and the concentration became

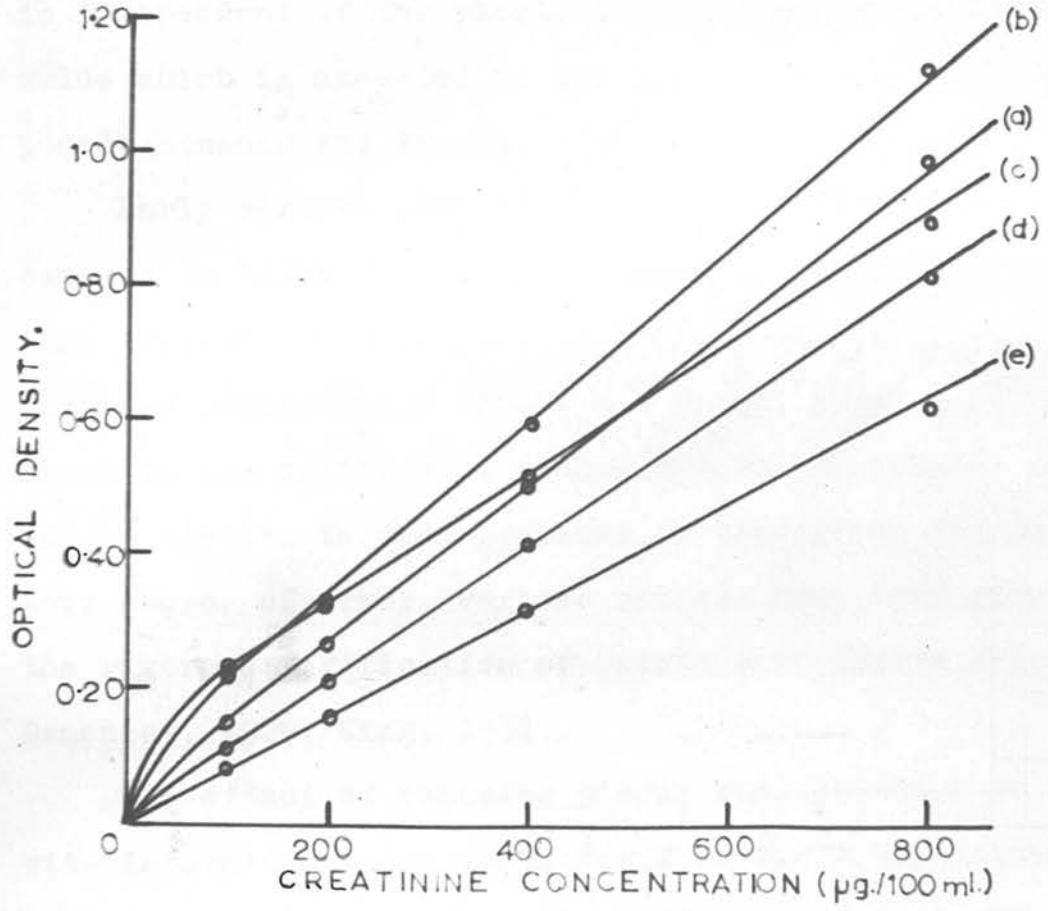

FIGURE 7. Optical density of alkaline creatinine picrate at various wavelengths.

4 ml. creatinine solution and 2 ml. alkaline picrate; colour developed for 20 min. at 20 C.; 20 mm. cells. (a) 480 mµ, (b) 490 mµ, (c) 500 mµ, (d) 510 mµ, (e) 520 mµ.

increasingly more linear although the optical density itself decreased after a slight rise. At 520 mμ the relationship was linear up to a concentration of 800 μg./100 ml. and at a concentration of 800 μg./100 ml. it deviated from linearity by only 2%. For this reason the wavelength of 520 mμ was used in this investigation.

Picric Acid.

The extinction coefficient of alkaline creatinine picrate is independent of the picric acid concentration above a certain value which is exceeded in all the methods which have been proposed (Bonsnes and Tausky, 1945).

Early workers noticed that picric acid which had been exposed to light (Hunter and Campbell, 1916), to high temperature (Wilson and Plass, 1917) or, in some instances, merely obtained commercially (Folin and Doisy, 1917) contained material which on the addition of sodium hydroxide produced an orange colour similar to that produced by creatinine and to eliminate this source of error, various methods have been proposed for the rigorous purification of picric acid (Folin and Doisy, 1917; Benedict, 1929; King, 1951).

The effect of exposing picric acid solution to daylight, with intermittent sunlight, for four weeks was examined. The optical densities of the colours developed when creatinine solutions were added to alkaline picrate made from this picric acid were compared with those developed with alkaline picrate made from freshly prepared picric acid. The optical density

of the alkaline picrate, i.e. reagent blank, made with light-exposed picric acid was nearly four times that of the alkaline picrate made with fresh picric acid yet the optical density of alkaline creatinine picrate was unaltered (Table 17).

The increase in optical density on the addition of creatinine to alkaline picrate made from impure picric acid may be so small compared to the optical density of the reagent blank that, with visual comparators, the accurate determination of small amounts of creatinine is difficult or impossible. Modern spectrophotometers, however, measure the increase in optical density directly so that using these instruments the optical density of the reagent blank is much less important and, further, since the optical density of the alkaline creatinine picrate is unaltered by the presence of the unknown impurity, rigorous purification of the picric acid would seem to be a less necessary procedure than has been previously believed.

Sodium Hydroxide and the effect of pH.

The extinction coefficients of alkaline picrate and of alkaline creatinine picrate depend on the pH of the final solution which, in turn, depends on the concentration of sodium hydroxide and on the acidity of the original solutions. The extinction coefficient of alkaline picrate is reported to be a direct function of the pH of the final solution while the extinction coefficient of alkaline creatinine picrate is reported to be an indirect function of the pH of the final solution

TABLE 17

The Effect on Picric Acid of Exposure to Light

4 ml. creatinine solution + 2 ml. alkaline picrate. Colour development for 20 mins. at 20°C. Optical densities measured at 520 mμ 20 mm. cells.

Creatinine Concentration	Colour obtained using purified picric acid		Colour obtained using light-exposed picric acid	
	Optical density	Optical density less reagent blank	Optical density	Optical density less reagent blank
μg./100 ml.				
0	0.056	-	0.202	-
200	0.214	0.158	0.360	0.158
400	0.367	0.311	0.510	0.308

(Bonsnes and Tausky, 1945; Roscoe, 1953).

The effect of acidity was examined by adding alkaline picrate a) to sulphuric acid solutions of varying concentration, and b) to creatinine solutions containing comparable amounts of sulphuric acid. On account of the strongly alkaline nature of the final solution it was considered impracticable to measure its pH directly and the optical densities of the developed colours have therefore been related to the titratable acidity of the fluids to which the alkaline picrate was added since it was this which determined the pH of the final solution.

The optical density of alkaline picrate decreased with increasing acidity of the sulphuric acid (Table 18) and it follows, therefore, that the reagent blank when alkaline picrate is added to an acid medium, is less than when added to water. On the other hand, the optical density of alkaline creatinine picrate increased as the acidity increased although the relative change was much less. It follows from this that the optical density of alkaline creatinine picrate is greater when the original solution containing the creatine is acid, e.g. serum filtrates prepared by method II, than when the original solution is neutral, e.g. water or serum filtrates prepared by method I.

Effect of temperature.

Many workers have noted that variation of the temperature at which the reaction was carried out affected the determination

TABLE 18

The Effect of Acidity on the Optical Density of Alkaline Picrate and Alkaline Creatinine Picrate

Alkaline picrate added to:- a) H_2O; b) creatinine solution 200 µg./100 ml; c) creatinine solution 400 µg./100 ml. - each containing various comparable quantities of H_2SO_4. Colour development for 20 min. at 20°C. Optical densities measured at 520 mµ. 20 mm. cells.

Titratable acidity of original solution.	Optical Density % of value in water		
(ml. 1N NaOH/100 ml.)	a	b	c
0.0	100	106	100
4.2	88	102.5	102
8.3	76	105	105.5
12.5	62	109	107

of creatinine.

The effect of temperature variation was examined and it was found that an increase in temperature causes an increase in the optical densities of alkaline picrate and of alkaline creatinine picrate and, furthermore, that this effect is reversible (Table 19). The temperature and, consequently, the optical density of the alkaline picrate in the reagent blank increases when the solution is repeatedly exposed to the heating effect of the light beam of the spectrophotometer (Haugen, 1951) so that water or air should be used as a reference optical density.

The reversibility of the effect of temperature becomes important when creatinine is determined using Lloyd's reagent according to the method of Haugen (1953) since it is not always possible to keep the solution containing the developing colour at a constant temperature, e.g. during centrifugation, and it may, therefore, be necessary to adjust the temperature of the solutions immediately before determination of their optical densities.

Colour Development.

After the addition of alkaline picrate to a pure creatinine solution the optical density of the colour developed reaches a maximum within a short time, 10-20 min., and remains constant for several hours (Hunter and Campbell, 1916; Bonsnes and Tausky, 1945; Hare, 1950; Haugen, 1953). When creatinine is originally present in an acid medium a longer time is required

TABLE 19.

The Effect of Temperature on the Optical Density of Alkaline Picrate and Alkaline Creatinine Picrate.

4.0 ml. creatinine solution + 2.0 ml. alkaline picrate. Colour developed for 20 min. Optical densities measured. at 520 mμ and expressed as % of value at 20°C. 20 mm. cells.

Temperature of colour development		15°C.	25°C.	15°C.	15°C.
Temperature of colour measurement		15°C.	25°C.	25°C.	Heated to 25°C. then cooled to 15°C.
	Creatinine Concentration μg./100 ml.				
Alkaline Picrate	0	96	125	123	98
Alkaline Creatinine Picrate	75	94	110	108.5	93
	150	94.5	110.5	109	95
	300	95	110	109	94

for the development of maximum optical density (Roscoe, 1953). In diluted urine the colour development is similar to that in pure creatinine solutions. In plasma or serum filtrates, however, a colour develops which does not reach a maximum optical density for several hours. This is believed to be due to the presence of one or more unknown chromogens, termed collectively non-creatinine chromogen (Hunter and Campbell, 1916; Hayman, Johnson and Bender, 1935; Hare, 1950, Haugen, 1953).

The rate of colour development in various media was examined (Fig. 8). In pure creatinine solution the optical density reached a maximum 16 min. after the addition of alkaline picrate and remained constant for longer than 1 hr. In diluted urine, and in the eluates obtained from Lloyd's reagent which had been shaken with pure creatinine solution, with serum filtrate or with diluted urine, the rate of colour development was similar to that in pure creatinine solutions and the optical density likewise remained at its maximum value for 1 hr. In untreated filtrates, however, and in filtrates which had been treated with the NC-bacteria, colour development was initially rapid and then continued to increase at a slower rate so that the maximum optical density was not reached in 1 hr.

Effect of other substances on the Jaffe reaction.

The optical density of alkaline picrate is variably affected by the presence of other substances. Richter (1944) and Lauson (1951) reported that glucose, which is itself chromogenic, depresses the optical density of alkaline picrate

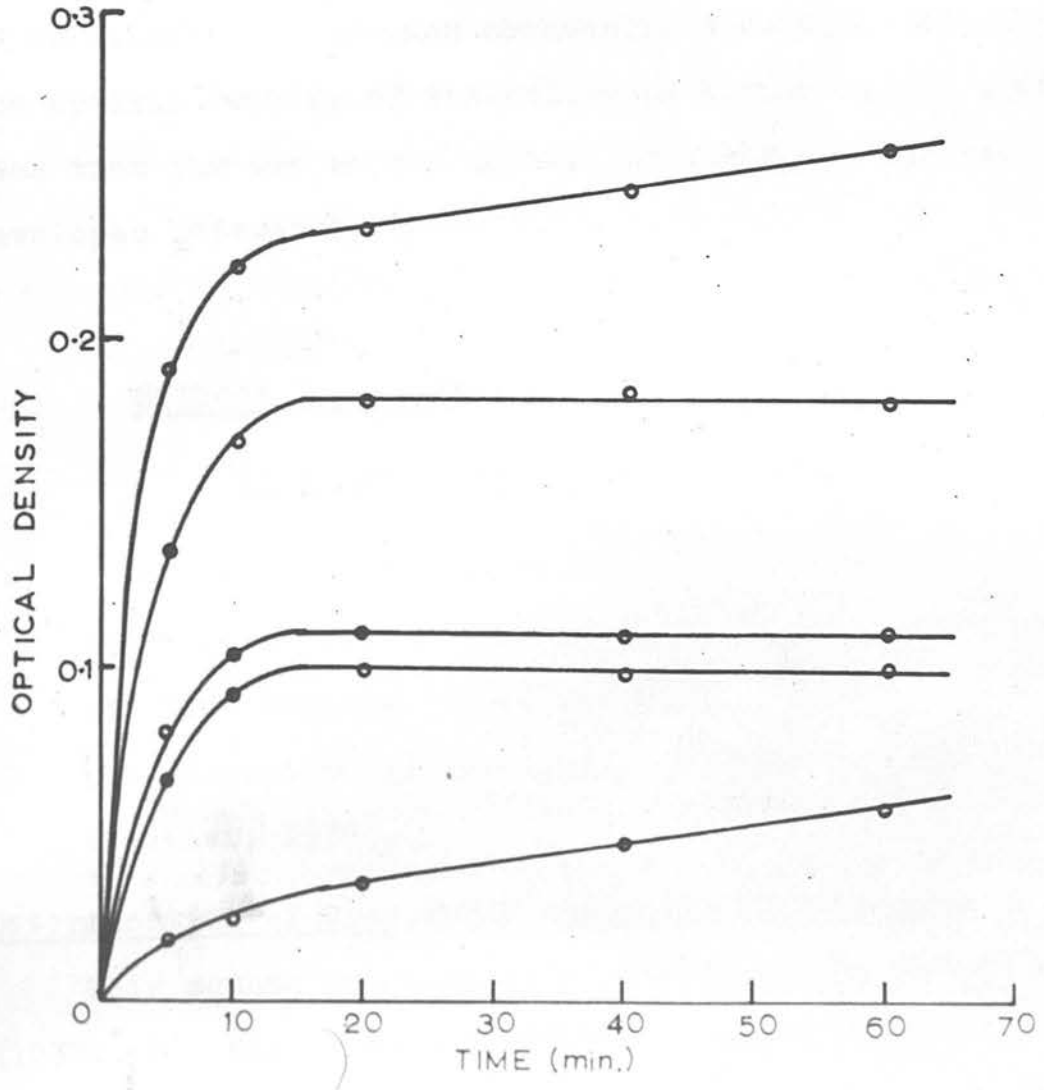

FIGURE 8. Rate of Colour Development on the addition of Alkaline Picrate to Various Solutions. 4 ml. Test Solution and 2 ml. alkaline picrate; colour developed at 20°C; Wavelength 520 mμ; 20 mm. cells.
(a) creatinine in water, 200 ug./100 ml; (b) diluted urine; (c) eluate from Lloyd's reagent, shaken with serum filtrate; (d) serum filtrate; (e) serum filtrate after incubation with NC-bacteria.

and this was confirmed. Other substances are reported to increase the optical density of creatinine picrate. That such an effect might occur in plasma filtrate was first suggested by Gaebler and Keltch, (1928) and, indeed, Lauson (1951) demonstrated that when alkaline picrate is added to a mixture of creatinine and the non-creatinine chromogen from plasma, the optical density of the colour developed by the mixture was less than the sum of the optical densities of the two colours developed separately.

Methods for Increasing the Specificity of the Jaffe reaction towards Creatinine.

These have been based on two principles:-

1) The destruction of creatinine by bacteria containing adaptive enzymes (Dubos and Miller, 1937).
2) The adsorption of creatinine on Lloyd's reagent (Gaebler, 1930).

Determination of Creatinine using the NC-bacteria.

This method has been extensively used by Miller and Dubos (1937a, b), Miller et al. (1939), Allinson (1945) and Miller and Miller (1951) who have justified its use on the following evidence.

1. The NC-bacteria quantitatively destroy creatinine added to plasma or serum filtrates or to diluted urine.

2. Creatinine is the only known chromogen which, being

present in plasma filtrates and destroyed by the NC-bacteria, could account for the disappearance of chromogen in filtrates treated with these bacteria.

The effect of the NC-bacteria on the chromogen in "neutral" (method I) and in "acid" (method II) serum filtrates and in diluted urines was examined, before and after the addition of creatinine. The "acid" filtrates were adjusted to pH 7 before incubation with the NC-bacteria.

Not all the chromogen originally present was destroyed by the bacteria, whereas creatinine added in concentrations from 100 - 250 µg./100 ml. was quantitatively destroyed (Table 20).

The effect on the reaction of increasing the number of bacteria was examined by doubling the volume of the bacterial suspension added to a "neutral" filtrate, removing aliquots of the incubating mixture at intervals, stopping the reaction by heating rapidly to 100°C. and determining the chromogen present in the aliquots. Although chromogen disappeared from the filtrate more rapidly the final amount of chromogen was the same as when the usual volume of bacterial suspension was added (Fig. 9).

This finding suggests that the NC-bacteria do not form an appreciable amount of the non-creatinine chromogen under the conditions used but, even with the other evidence, does not exclude the possibility that one or more unknown chromogens are also destroyed.

TABLE 20

Destruction by NC-bacteria of Creatinine added to Serum Filtrates and to Diluted Urine.

5.0 ml. serum filtrate or diluted urine, 0.2 ml. phosphate buffer, 0.5 ml. bacterial suspension; incubated 37°C for 60 min. The concentration of added creatinine ranged from 100-250 µg./100 ml.

Medium	Initial total chromogen (as creatinine)	Non-creatinine chromogen (as creatinine)	Non-creatinine chromogen after added creatinine (as creatinine)
	µg./100 ml.	µg./100 ml.	µg./100 ml.
Serum Filtrate Method I	159	36	36
	168	28	27
	206	36	32
	155	26	26
	156	30	26
	187	51	37
	175	37	32
	160	33	35
Serum Filtrate Method II (adjusted to pH 7.0)	247	86	72
	188	42	47
	216	60	50
Diluted Urine	222	2	2
	184	2	8
	135	10	16
	202	5	3
	142	9	10
	205	9	8
	143	9	9

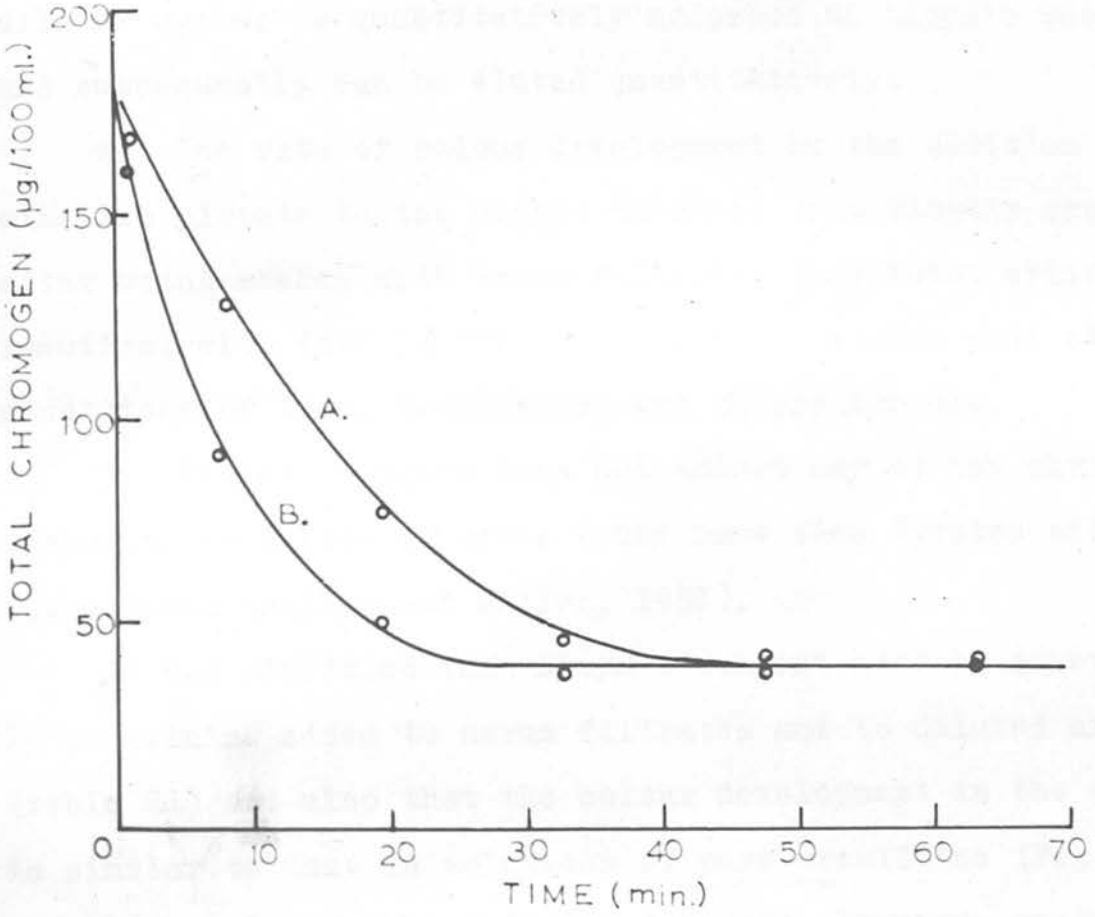

FIGURE 9. Rate of disappearance of chromogen from serum filtrate incubated with different amounts of NC-bacteria.

(a) serum filtrate 25 ml.; phosphate buffer 1 ml., water 2.5 ml., bacterial suspension 2.5 ml.
(b) serum filtrate 25 ml., phosphate buffer 1 ml., bacterial suspension 5 ml.

The adsorption method using Lloyd's reagent.

This has been used with different modifications, by Gaebler (1930), Borsook, (1935), Danielson (1936), Brod and Kotatko (1949), Hare (1950) and Haugen and Blegen (1953), who have justified its use on the following evidence.

1. Creatinine added to plasma or serum filtrates and to diluted urines is quantitatively adsorbed on Lloyd's reagent and subsequently can be eluted quantitatively.

2. The rate of colour development on the addition of alkaline picrate to the eluate obtained from Lloyd's reagent after being shaken with serum filtrates or diluted urines is identical with that in pure creatinine solutions when similar conditions of time, temperature and pH are imposed.

3. Lloyd's reagent does not adsorb any of the chromogen occurring in filtrates after these have been treated with the NC-bacteria (Miller and Miller, 1951).

It was confirmed that Lloyd's reagent adsorbs quantitatively creatinine added to serum filtrates and to diluted urine (Table 21) and also that the colour development in the eluate is similar to that in solutions of pure creatinine (Fig. 8).

The whole of this evidence does not, however, exclude the possibility that Lloyd's reagent adsorbs other chromogens, nor the possibility that some creatinine is formed from a precursor during the process of adsorption and elution as was suggested by Gaebler (1930). It must be noted, too, that all samples of Lloyd's reagent have not equal ability to adsorb creatinine

TABLE 21

The Recovery of Creatinine added to Serum Filtrates and to Diluted Urine using Lloyd's Reagent.

100 mg. Lloyd's reagent, 5.0 ml. serum filtrate or diluted urine, 0.5 ml. oxalic acid. Elution with 7.5 ml. alkaline picrate - diluted 1 in 3 with water. Analyses in duplicate.

Medium	Initial Concentration of creatinine	No. of specimens	Amount added	Recovery Mean & range
	µg./100 ml.		µg./100 ml.	%
Serum filtrate Method I	67 - 137	7	100 - 200	99.9 (96.6-101.5)
Serum filtrate Method II	142 - 246	4	200	103.8 (100.5-108.1)
Diluted Urine	129 - 209	5	100	99.2 (95.8-105.3)

(Haugen and Blegen, 1953). Nevertheless, a satisfactory sample of this reagent is superior to other adsorption reagents including kaolin, synthetic magnesium trisilicate and ion-exchange resins, all of which were found to suffer from the disadvantage of inefficient adsorption or of high and variable reagent blanks.

The Recovery of Creatinine added to Plasma or Serum, or to Protein-free Filtrates prepared from these.

Protein Precipitation.

Various procedures which have been used in the preparation of protein-free filtrates of plasma or serum have been reported to give different values for the recovery of added creatinine. These procedures may be conveniently divided into two groups:- those which provide a "neutral" filtrate, i.e. with a pH greater than 2.5, and negligible titratable acidity, and those which provide an "acid" filtrate with a lower pH and considerable titratable acidity. Filtrates obtained by using sodium tungstate (10% w/v) and sulphuric acid (0.66N) in equal volumes, e.g. Method I, by using cadmium hydroxide or by ultrafiltration belong to the first group. Filtrates obtained by using sodium tungstate and larger proportions of sulphuric acid, e.g. Method II, or by using trichloracetic acid belong to the second group.

When mixed, equal volumes of sodium tungstate (10% w/v) and sulphuric acid give an acid solution. The excess sulphuric acid is partly neutralised by the proteins present in the plasma

authors put forward the suggestion that some of the creatinine was retained on the precipitated protein.

Haugen (1953) found that four different methods of producing tungstic acid filtrates gave mean values for the recovery of creatinine added to plasma of 78% (68-84%), 85% (79-91%), 94% (84-103%) and 100% (94-100%) respectively. He too found the mean recovery of creatinine added to filtrates to be higher - 99%, 100%, 100% and 98%, respectively. Similarly Roscoe (1953) found that "neutral" tungstic acid filtrates gave a mean recovery of creatinine added to serum of 86% while "acid" tungstic acid filtrates gave a mean recovery of 100%. These data relating to the recovery of creatinine have been summarised (Table 22).

Lauson (1951) examined this problem further, by comparing the recovery of creatinine added to plasma with the recovery from "neutral" tungstic acid filtrates of the same plasma and found that the recovery from plasma was on the average 94% (90-98%) of that from the filtrates. He also noted that the recovery from plasma varied with the plasma protein concentration, in that the recovery from plasma with a low protein concentration was less than that from a plasma with a high protein concentration. This finding is apparently contrary to the conclusions of Camara et al. and suggest that any adsorption of creatinine on the precipitated protein is not dependent solely on the amount of precipitate present.

Hare (1950) using Lloyd's reagent with trichloracetic acid filtrates obtained recoveries of creatinine added to serum

TABLE 22.

Recovery of Creatinine using Various Types of Tungstic Acid Filtrates.

Plasma or Serum	Water	Sodium tungstate (10% w/v)	Sulphuric Acid (0.66N)	Mean pH (present author)	Recovery of Creatinine (various authors)		
					From Plasma or Serum	from Filtrates	Ref.
vol.	vol.	vol.	vol.		%	%	
					100	-	(1)
					100	98	(3)
1.0	1.5	0.5	1.0	1.58	99	-	(4)
(Method II)					100	-	(5)
					100	104	(6)
1.0	2.0	1.0	1.0	2.52	78	99	(3)
					88	-	(4)
1.0	7.0	1.0	1.0	2.62	88	100	(2)
					85	96	(3)
					90	-	(4)
					90	-	(1)
1.0	2.0	0.5	0.5	4.00	78	95	(2)
					86	98	(5)
1.0	3.0	0.5	0.5	4.06	89	100	(6)
(Method I)					97	-	(4)
1.0	8.0	0.5	0.5	4.16	94	100	(3)

Ref. (1) Brod & Sirota, (1948); (2) Camara et al., (1951); (3) Haugen, (1953); (4) Mandel & Jones, (1953); (5) Roscoe, (1953); (6) Present investigation.

of 98-101%. Mandel and Jones (1953), also using Lloyd's reagent, obtained with trichloracetic acid filtrates a mean recovery of 102% (98-111%). With 1 in 10 "neutral" tungstic acid filtrates the mean recovery was 91% (85-97%); with 1 in 5 "neutral" tungstic acid filtrates 94% (90-96%); with "acid" tungstic acid filtrates 99% (98-102%) and with cadmium hydroxide filtrates 93% (88-99%).

Recoveries obtained in the present investigation.

The recovery of creatinine was examined using both "neutral" and "acid" filtrates. Creatinine was determined as total chromogen using the NC-bacteria and using Lloyd's reagent. The mean recoveries of creatinine added to serum determined as total chromogen were higher with "acid" filtrates than with "neutral" filtrates at all levels of added creatinine. When 1.0 mg./100 ml. was added to serum the mean recovery using "neutral" filtrates was 89%, while using acid filtrates it was 100% (Table 23). At a level of 10 mg./100 ml. of added creatinine the recoveries were 92% and 97%, respectively. At a level of 100 mg./100 ml. added creatinine the recoveries were 92% and 96%, respectively.

Using the NC-bacteria the mean recovery of creatinine added to serum (1.0 mg./100 ml.) was 91% with "neutral" filtrates and 100% with "acid" filtrates. The corresponding figures using Lloyd's reagent were 94% and 98%, respectively.

The recovery of creatinine added to serum filtrates was also examined. The mean recoveries of "creatinine" added to

TABLE 23. Recovery of Creatinine added to Serum

Results expressed as mean percentage of added creatinine.

Creatinine added	"Neutral" Filtrate		
	Total Chromogen	NC-bacteria	Lloyd's reagent
mg./100 ml.			
1.0	88.8 S.D. = 2.8 n = 14	90.8 S.D. = 3.7 n = 6	93.9 S.D. = 1.9 n = 6
10.0	91.5 range 86-97 n = 4	---	---
100.0	92.0 range 88-97 n = 4	---	---
	"Acid" Filtrate		
1.0	99.7 S.D. = 3.9 n = 12	100.0 S.D. = 6.8 n = 6	97.6 S.D. = 2.4 n = 5
10.0	96.7 range 94-99 n = 3	---	---
100.0	96.3 range 99-98 n = 3	---	---

"neutral" and to "acid" filtrates were, determined as total chromogen, 100% and 104%, respectively (Table 24). With Lloyd's reagent the figures were exactly similar, i.e. 100% and 104%, respectively. No recoveries of creatinine added to serum filtrates were carried out using the NC-bacteria since it had already been shown that the bacteria quantitatively destroy creatinine in serum filtrates.

The effect of the pH of the filtrate on the recovery of creatinine added to serum or to serum filtrates was examined further by adding various quantities of sulphuric acid to the protein precipitating mixture and determining the recovery of creatinine added originally to the sera and that of creatinine added to the filtrates. Minimum recovery (85-90%) from serum was obtained when the pH of the filtrate had a value of about 3.3 (Fig. 10A). Recoveries were virtually 100% at pH 2.0 and 95% at pH 4.5. Recoveries from filtrates averaged 100% and were independent of pH except in filtrates with pH less than 2.0 in which recoveries tended to be slightly greater than 100% (Fig. 10B).

When the mean recoveries of creatinine added to plasma or serum reported by other workers are grouped according to the type of filtrate used and the latter are arranged according to pH (as determined in the present investigation), there is evident the same tendency for the recovery to be minimal at a pH intermediate between that of filtrates prepared by method I and method II (Table 22).

TABLE 24. Recovery of Creatinine added to Serum Filtrate.

Results expressed as a percentage of added creatinine.

Creatinine added	Neutral Filtrate		Acid Filtrate	
	Total Chromogen	Lloyd's Reagent	Total Chromogen	Lloyd's Reagent
µg./100 ml.				
200-400	100.0 range 95-102 n = 5	99.9 range 97-102 n = 7	103.8 range 99-108 n = 6	103.8 range 100-108 n = 4

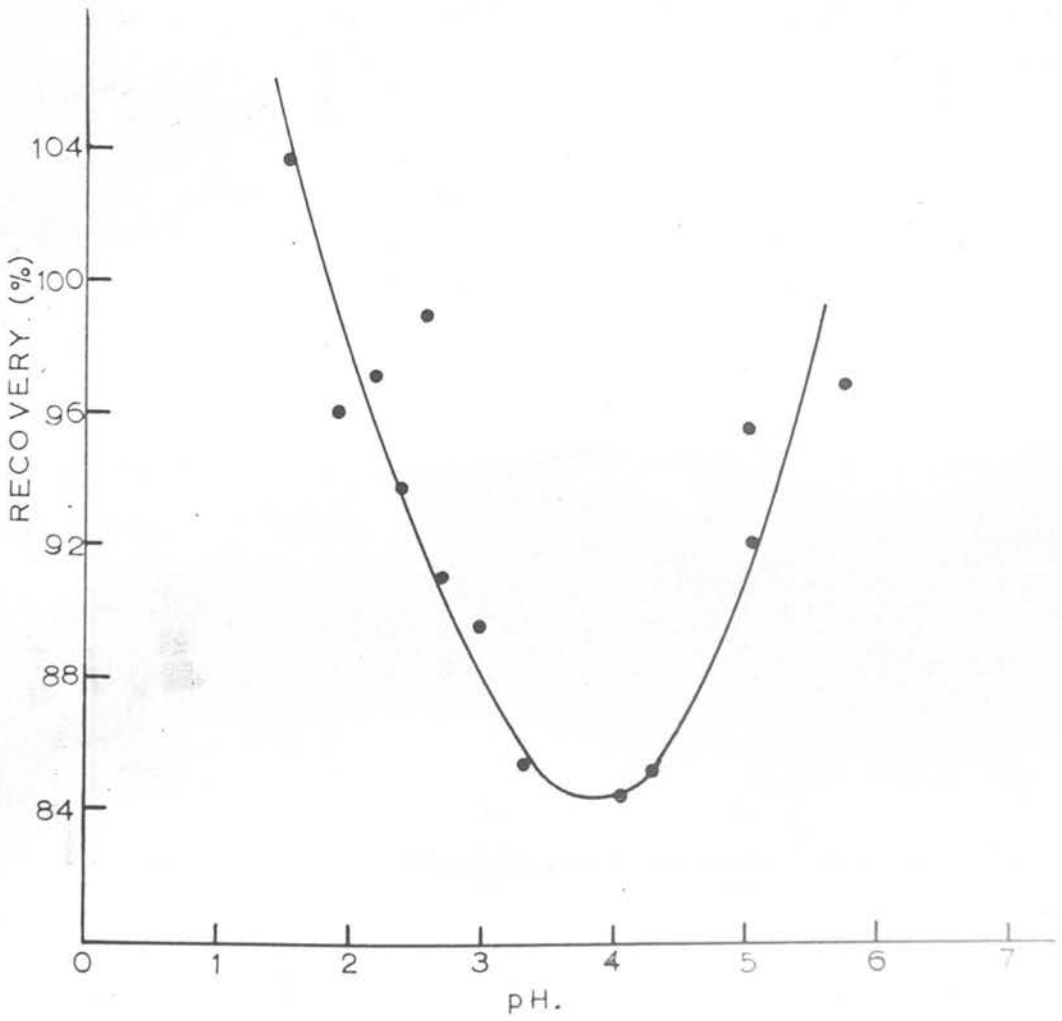

FIGURE 10A. The effect of pH of serum filtrate on the recovery of creatinine added to serum.

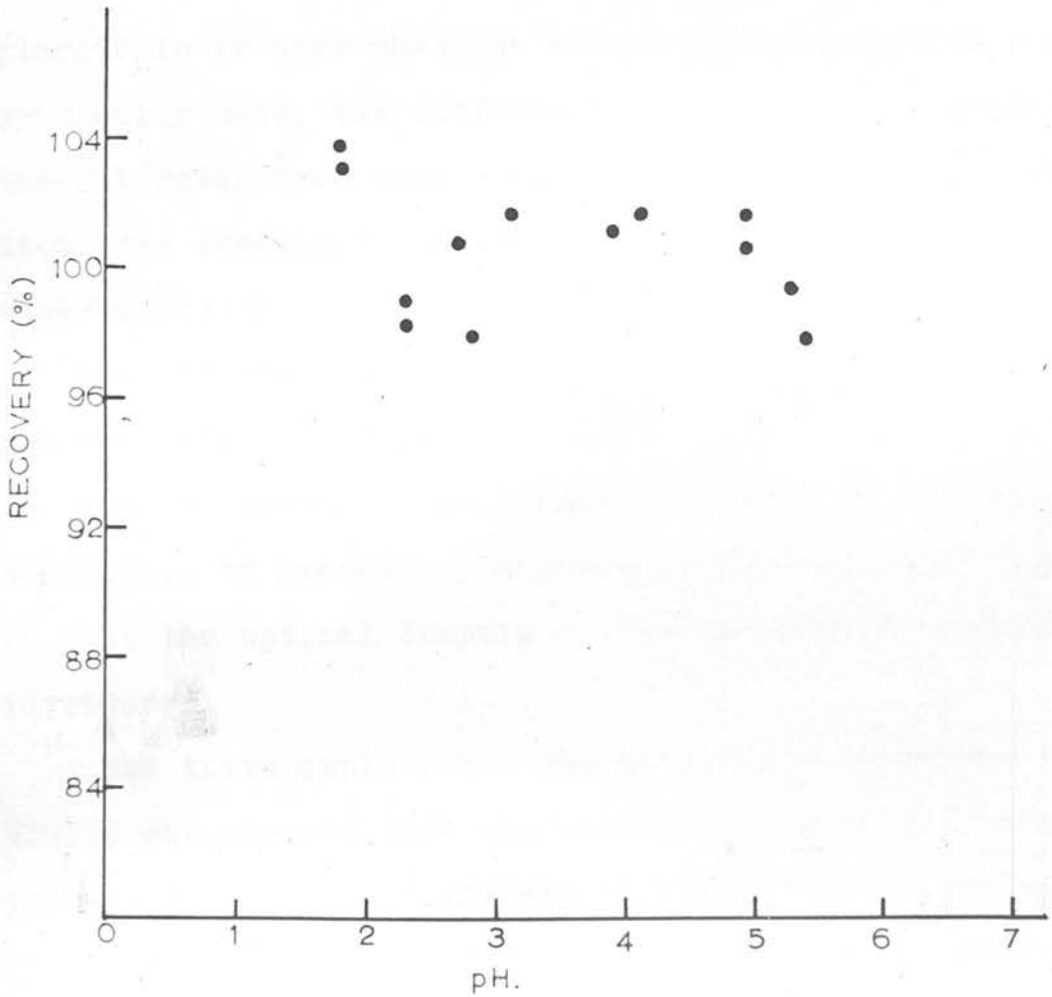

FIGURE 10B. The effect of pH of serum filtrate on the recovery of creatinine added to serum filtrate.

Discussion.

Three explanations can be offered for the finding of higher recoveries of creatinine added to serum using "acid" filtrates.

1. The colours developed in serum filtrates were compared with those developed from standard creatinine solutions in water. Since the optical density of alkaline creatinine picrate is greater when the colour is developed in a solution originally acid, the difference in the optical densities of the filtrates from sera with and without added creatinine (i.e. the recovery), is greater in the "acid" filtrates. In other words the different recoveries are apparent and not real.
2. Loss of creatinine may occur as a result of adsorption on the precipitated protein.
3. The creatinine is modified in some way during the precipitation of protein by methods giving "neutral" filtrates so that the optical density of the alkaline creatinine picrate is reduced.

The third explanation was originally suggested by Roscoe (1953) who claimed that recoveries with neutral filtrates were increased if the filtrate was diluted prior to the development of the colour. This finding was not, however, confirmed and furthermore, the recovery of large amounts of creatinine (10 and 100 mg./100 ml.) added to serum, which necessarily involved considerable dilution of the filtrate still gave mean recoveries of only 92% when "neutral" filtrates were used.

It would seem likely, however, that both factors involved in the first and second explanations contribute to the observed difference in recoveries. The first is undoubtedly responsible for part of the increased recovery noted when "creatinine" is determined as total chromogen and is consistent with the observation that recoveries of creatinine added to "acid" filtrates are occasionally greater than 100% (Table 24 and Fig. 10B). However the acidity of the filtrate cannot be solely responsible for the increased recoveries obtained since with Lloyd's reagent the colours in the eluates are all developed under the same conditions and still the "acid" filtrates give better recoveries than the "neutral" filtrates. It is concluded, therefore, that loss of creatinine by adsorption on the precipitated protein does occur, as was originally suggested by Camara et al. However, because of the difficulty of obtaining the protein precipitate sufficiently free from filtrate it was not found possible to test this suggestion directly.

The direct dependence of the recovery of creatinine added to plasma on the protein concentration of the plasma, as reported by Lauson (1951) is consistent with the above findings, for decrease in the plasma protein concentration causes a decrease in the pH of the filtrate and, provided this is still above the value giving minimum recovery decreased recovery occurs. (Fig. 10A).

The "Creatinine" Content of Plasma or Serum Determined by Various Methods

In comparing the results obtained with different methods the specificity of the method in relation to the possible presence of different amounts of non-creatinine chromogen must be considered in addition to the effect of the pH of the protein-free filtrate on the extinction coefficients of alkaline picrate and alkaline creatinine picrate and on the possible loss of creatinine by retention on the precipitated protein.

Results obtained by previous workers.

Discrepancies between the results of different methods were noted by many early workers. Ferro-Luzzi and co-workers (1934, 1935) reported that zinc hydroxide filtrates contained less total chromogen than "neutral" tungstic acid filtrates. Danielson (1936) reported that "neutral" tungstic acid filtrates contained essentially the same amount of total chromogen as ultrafiltrates. Smith, Finkelstein and Smith (1940) reported that "neutral" tungstic acid filtrates contained less total chromogen than ferric carbonate filtrates. Brod and Sirota (1948), however, reported that "neutral" and "acid" tungstic acid filtrates contained the same amount of total chromogen although the recoveries of creatinine added to plasma using these filtrates were 90% and 100%, respectively. Camara et al. (1951) reported that 1 in 4 "neutral" tungstic acid filtrates contained 21% more total chromogen than 1 in

10 "neutral" tungstic acid filtrates. The recoveries of creatinine added to plasma, however, were only 78% and 88%, respectively.

Haugen (1953) determined the total chromogen in sera using various tungstic acid filtrates and found the mean values obtained with three "neutral" filtrates were 97%, 104% and 100% of the mean value obtained with "acid" filtrates. Moreover, the mean values obtained for the total chromogen content bore no consistent relation to the different mean recoveries obtained for creatinine added to serum using the respective filtrates. Measuring the optical densities of the colours developed in the filtrates at 15 min. and at 24 hr. after the addition of the alkaline picrate he found that the increase in colour during this time was not the same in each filtrate and concluded that the different filtrates contained different amounts of non-creatinine chromogen and that this was responsible for the differences on total chromogen contents.

Comparison of the results obtained using the allegedly specific methods have shown, however, that different amounts of non-creatinine chromogen can be only partly responsible for differences in total chromogen content. Miller and Miller (1951), using the NC-bacteria, reported that 1 in 10 "neutral" tungstic acid filtrates gave values for the "creatinine" content of plasma on the average 9% higher than 1 in 4 "neutral" tungstic acid filtrates and Mandel et al. (1953), using Lloyd's reagent, reported that the "creatinine" contents of trichloracetic acid filtrates of plasma were 4-10% higher than those

of "neutral" tungstic acid filtrates and 7-12% higher than those of cadmium hydroxide filtrates.

Comparative results obtained in the present investigation.

The "creatinine" contents of sera were examined using "neutral" and "acid" filtrates and determining "creatinine" as total chromogen, with the NC-bacteria and with Lloyd's reagent (Table 25). Since the sera were not always analysed by all three methods the figures in this Table can be used only to compare "acid" and "neutral" filtrates. There was no difference between the mean value for the total chromogen content in "acid" and in "neutral" filtrates, the mean ratio being 1.02.

Since, however, the optical density of alkaline picrate and of alkaline creatinine picrate depend on pH, i.e. on the acidity of the original solution, the total chromogen contents of a further series of sera were determined with the "acid" filtrates neutralised, i.e. adjusted to pH 7, before developing the colour[1]. The mean value for the total chromogen content of these neutralised "acid" filtrates was 5% (2-13%) higher than that of "neutral" filtrates. On this basis the mean ratio of total chromogen in "acid" filtrates to that in "neutral" filtrates is 1.05.

Using the NC-bacteria the mean "creatinine" content, i.e. the difference between the chromogen content before and after

(1) So-called "neutral" filtrates are not actually at pH 7 but the titratable acidity present is negligible so that neutralisation is unnecessary.

TABLE 25. "Creatinine" Content of Normal Sera. Comparison of "Neutral" and "Acid" Filtrates.

The means of the results obtained by different methods can only be compared in different filtrates since the sera were not always analysed by all three methods.

Filtrate	Total Chromogen	NC - bacteria		Lloyd's reagent
		Non-creatinine Chromogen	Total non-creatinine Chromogen	
	mg./100 ml.	mg./100 ml	mg./100 ml	mg./100 ml.
"Acid" (unneutralised for total chromogen)	0.874 S.D. = 0.189 n = 14	0.267 S.D. = 0.071 n = 8	0.601 S.D. = 0.154 n = 8	0.756 S.D. = 0.086 n = 10
"Neutral"	0.886 S.D. = 0.173 n = 14	0.185 S.D. = 0.051 n = 8	0.715 S.D. = 0.154 n = 8	0.716 S.D. = 0.082 n = 10
"Acid" / "Neutral"	1.02 S.D. = 0.049 n = 14 p* 0.4	0.695 S.D. = 0.073 n = 8 p 0.01	0.833 S.D. = 0.124 n = 8 p 0.05	1.05 S.D. = 0.052 n = 10 p = 0.05

*Probability of mean ratio differing from 1.0 by chance alone.

incubation with the bacteria, was in "acid" filtrate 17% lower than in "neutral" filtrates, the mean ratio being 0.83 and this difference was statistically significant. In this series of results the total chromogen content of the "acid" filtrate was determined without prior neutralisation and the figures for "creatinine" content of "acid" filtrates should, therefore, be increased by approximately 5%. However, if this approximate correction is made, the "creatinine" content of the "acid" filtrates is still significantly lower than that of the "neutral" filtrates.

Using Lloyd's reagent the mean "creatinine" content in "acid" filtrates was 5% higher than in "neutral" filtrates, the mean ratio being 1.05 and this difference was statistically significant.

With certain sera it was possible to compare the results obtained by the three techniques in both "acid" and "neutral" filtrates (Table 26). The "creatinine content determined with the NC-bacteria or with Lloyd's reagent was always less than the total chromogen content. The mean ratio of "creatinine" determined with the NC-bacteria to total chromogen was 0.78 in "neutral" filtrates and 0.67 in "acid" filtrates. Using Lloyd's reagent these ratios were 0.81 and 0.87 (corrected ratio 0.82)*, respectively.

* Corrected since total chromogen content of "acid" filtrate was determined without prior neutralisation.

TABLE 26. "Creatinine" Content of Normal Sera. Comparison of Methods.

"Creatinine" determined as total chromogen, with the NC - bacteria and with Lloyd's reagent in "neutral" and "acid" filtrates. Results expressed as mean ratios of results.

Filtrate	NC - bacteria / Total chromogen	Lloyd's reagent / Total chromogen	NC - bacteria / Lloyd's reagent
"Neutral"	0.783 S.D. = 0.051 n = 10 p* 0.01	0.810 S.D. = 0.039 n = 10 p 0.01	0.953 S.D. = 0.087 n = 12 p 0.3
"Acid" (unneutralised for total chromogen).	0.672 S.D. = 0.126 n = 8 p* 0.01	0.866 S.D. = 0.038 n = 10 p 0.01	0.823 S.D. = 0.097 n = 8 p 0.02
"Acid" (corrected)	0.67 p* 0.01	0.82 p 0.01	0.87 p 0.05

*Probability of mean ratio differing from 1.0 by chance alone.

Comparing the two "specific" methods the mean "creatinine" content obtained with the NC-bacteria in "neutral" filtrates was lower than that obtained with Lloyd's reagent, the mean ratio being 0.95, but this difference was not statistically significant. In "acid" filtrates the mean value obtained with the NC-bacteria was 13% lower than that obtained with Lloyd's reagent, the mean ratio being 0.82 (corrected ratio 0.87). This difference, due to the apparent smaller "creatinine" content of acid filtrates as determined with the NC-bacteria and to the apparent larger "creatinine" content obtained with Lloyd's reagent, was statistically significant.

The anomalous finding of greater amounts of non-creatinine chromogen, as determined by the NC-bacteria, in "acid" filtrates was investigated further by determining the chromogen content of "neutral" and "acid" filtrates prepared from plasma or serum, which had been dialysed in cellophane (Visking) tubes for 6 days against running tap-water. In some cases creatinine was added to the serum or plasma before dialysis. "Creatinine" was determined as total chromogen, with the NC-bacteria and with Lloyd's reagent.

The mean total chromogen content using "neutral" filtrates was 0.13 mg./100 ml. dialysed plasma or serum, while with neutralised "acid" filtrates a mean value of 0.14 mg./100 ml. was obtained (Table 27). These values are slightly less than the mean non-creatinine chromogen content of normal serum as given by the difference between the values obtained

TABLE 27. Chromogen in Dialysed Sera or Plasmas.

Results in terms of creatinine per 100 ml. dialysed serum or plasma.

Material	Total Protein N x 6.25	"Neutral" Filtrate			"Acid" Filtrate"		
		Total Chromogen	NC-bacteria	Lloyd's Reagent	Total Chromogen	NC-bacteria	Lloyd's Reagent
	g./100 ml.	mg./100 ml	mg./100 ml	mg./100 ml	mg./100 ml	mg./100 ml	mg./100 ml.
Serum A	3.1	0.14	-	0.08	0.14	-	0.04
Serum B	4.4	0.16	0.18	0.06	0.17	0.30	0.04
Serum C	5.1	0.16	-	0.00	0.10	-	0.00
Serum C + 10 mg./100 ml. Creatinine	4.8	0.10	-	0.00	0.08	-	0.00
Serum C + 20 g/100 ml. Creatinine	5.0	0.16	-	0.00	0.20	-	0.00
Plasma D	4.5	0.09	0.15	0.05	0.13	0.25	0.06
Plasma D + 10 g./100 ml. Creatinine	4.4	0.13	0.20	0.06	0.16	0.23	0.05

as total chromogen and that with Lloyd's reagent. Using the NC-bacteria the mean non-creatinine content was 0.18 mg./100 ml. and 0.26 mg./100 ml. with "neutral" and "acid" filtrates, respectively, values practically identical with those obtained for undialysed sera (Table 25). Using Lloyd's reagent the mean chromogen content was 0.03 mg./100 ml. with "neutral" filtrates and 0.04 mg./100 ml. with "acid" filtrates.

Since the total chromogen contents in the dialysis residua from plasma or serum to which creatinine had been added were practically the same as those from plasma or serum without added creatinine, and since none of this chromogen was destroyed by the NC-bacteria it was concluded that none of the chromogen present in these filtrates was, in fact, creatinine.

Discussion.

The finding of apparently identical amounts of total chromogen in "neutral" and in unneutralised "acid" filtrates is in agreement with the findings of Brod and Sirota (1948) and of Haugen (1953), although this is inconsistent with the increased recoveries obtained with "acid" filtrates. This anomalous finding with unneutralised "acid" filtrates is most likely due to the interaction of three factors:- a) the reagent blank, i.e. the optical density due to the alkaline picrate is really less in the final solution obtained from "acid" filtrates than in those obtained from the creatinine standard solutions and from "neutral" filtrates; b) the optical density of alkaline creatinine picrate is greater the more acid the original solution (Table 18); and c) "acid" filtrates actually

contain more creatinine than "neutral" filtrates from the same serum as indicated by the recovery experiments. When the effect of the acidity is eliminated either by neutralisation of the "acid" filtrates or by using Lloyd's reagent the anomaly disappears.

Considering the two specific methods it is evident that they give a more correct value for the creatinine content of serum filtrates than determinations of total chromogen. It is not easy, however, to draw definitive conclusions as to the relative merits of these two methods. It is clear that the NC-bacteria are capable of destroying creatinine completely and that Lloyd's reagent can adsorb creatinine completely. However these two techniques give significantly different results when applied to "acid" filtrates although with "neutral" filtrates agreement is good. The difference using "acid" filtrates appears to be largely due to the anomalous results obtained with the NC-bacteria which indicate apparently greater quantities of non-creatinine chromogen both in "acid" filtrates from which the creatinine has been removed by the incubation with the bacteria and in "acid" filtrates of dialysed serum from which the creatinine has already been removed. On the whole, therefore, it would seem that the Lloyd's reagent method gives the more reliable results and it has the additional advantage of greater simplicity.

Determination of "Creatinine" in Urine.

Recoveries of added creatinine.

In contrast to recoveries of creatinine added to plasma or serum, it has generally been reported that the recovery of creatinine added to urine is quantitative.

The mean recoveries of creatinine added to ten different diluted urines were as follows. Determined as total chromogen - 101.7% (100-104%); with the NC-bacteria - 101.4% (95-106%); and with Lloyd's reagent - 99.2% (95-103%).

"Creatinine" contents of urine determined by different methods.

The "creatinine" content of urines were examined using the three methods. The mean "creatinine" content determined with the NC-bacteria was 3% lower than the mean total chromogen content, the mean ratio being 0.97 (Table 28) and this difference was statistically significant. The mean "creatinine" content determined with Lloyd's reagent was 6% lower than the mean total chromogen content the mean ratio being 0.95 and this difference was also statistically significant.

The mean creatinine content determined with the NC-bacteria was 3% higher than the mean creatinine content determined with Lloyd's reagent the mean ratio being 0.97 but this difference was not statistically significant.

Accuracy of Determinations in Serum or Urine.

The standard deviation of determination in serum were found to be as follows:- Total chromogen - neutral filtrate

TABLE 28.

"Creatinine" Content of Diluted Normal Urine. Comparison of Methods.

"Creatinine" determined as total chromogen, with the NC - bacteria and with Lloyd's reagent. Results expressed as mean contents No. of urines = 12.

Total chromogen	NC - bacteria		Lloyd's reagent.
	Non-creatinine chromogen	Total less non-creatinine chromogen	
µg./100 ml	µg./100 ml	µg./100 ml	µg./100 ml
235	8	229	223

Results expressed as ratios of "creatinine" contents.

NC - bacteria / Total chromogen	Lloyd's reagent / Total chromogen	NC - bacteria / Lloyd's reagent
0.967	0.948	0.975
S.D. = 0.025	S.D. = 0.025	S.D. = 0.05
n = 12	n = 12	n = 12
p* 0.01	p 0.01	p 0.8

*Probability of mean ratio differing from 1.0 by chance alone.

1.0% (n=14); Lloyd's reagent - neutral filtrate 2.0% (n=12); Lloyd's reagent - acid filtrate 1.5% (n=12).

In undiluted urines the standard deviations were found to be total chromogen 0.9% (n=20); Lloyd's reagent 1.6% (n=12).

Serum "Creatinine" in persons with renal disease.

A short series of determinations were made on sera from persons with various degrees of renal dysfunction. Total chromogen content and non-creatinine chromogen content, using NC-bacteria were determined in "neutral" tungstic acid filtrates (Table 29).

The relative amount of non-creatinine chromogen was found to be less in sera containing a high total chromogen content than in normal sera. This finding is in agreement with the findings of Brod and Kotatko (1949), and Miller, Leaf, Mamby and Miller (1952a).

The Nature of the Non-Creatinine Chromogen in Plasma and Urine.

This was not investigated in detail, although a few experiments were carried out to investigate its behaviour towards the procedures used in the determination of creatinine. It seems most likely that there are present in plasma and urine more than one unknown chromogen.

Before discussing its nature it is convenient to summarise its properties:-

TABLE 29 "Creatinine" Contents of Sera from Persons with Various Diseases.

Diagnosis	Sex	Age	Total Chromogen	Non-creatinine Chromogen (NC-bacteria)	True Creatinine	Creatinine as % of total Chromogen
			mg./100 ml	mg./100 ml	mg./100 ml	
Hypertension	F	-	0.67	0.23	0.44	66
Hypopituitarism	M	56	0.70	0.18	0.52	74
Hypertension	F	43	0.80	0.12	0.68	85
Hypopituitarism	M	60	0.85	0.15	0.70	82
Hypertension	M	-	0.97	0.23	0.74	77
Hypopituitarism	M	50	0.96	0.26	0.70	73
Hypertension	M	76	1.08	0.18	0.90	83
Hypopituitarism	F	42	1.13	0.2	0.92	81
Hyperparathyroidism	F	-	1.50	0.25	1.25	83
Chronic nephritis	M	64	1.70	0.15	1.55	91
Chronic nephritis	M	-	4.8	0.92	3.88	81
Chronic nephritis	M	-	6.75	1.15	5.60	83
Hypertension) Chronic) nephritis)	M	26	15.6	1.03	14.57	94
Chronic pyelonephritis	M	-	24.5	2.6	21.9	89

1. It reacts with alkaline picrate to give a colour which does not attain maximum optical density for at least 1 hr.
2. It is not adsorbed, at least completely, on Lloyd's reagent.
3. It is not destroyed, at least completely, by the NC-bacteria.
4. It is present in plasma and to a smaller extent in urine. It is reported to be present in erythrocytes but not in C.S.F. (Miller and Dubos, 1937b).
5. Only a small fraction can be removed from serum or plasma by prolonged dialysis.

These findings, as far as plasma or serum is concerned, refer strictly to "protein-free" filtrates of these fluids. Protein is itself a chromogen and would form part of the non-creatinine chromogen if it were incompletely precipitated in the preparation of protein-free filtrate. Christensen and Lynch (1946) reported that tungstic acid and trichloracetic acid filtrates of plasma contained non-diffusible -amino nitrogen, presumably protein, but the amounts found were only sufficient to account for a small fraction of the non-creatinine chromogen. On the other hand, the results of the dialysis experiments suggest that a large fraction of the non-creatinine chromogen is non-diffusible and this is in agreement with the finding in urine of amounts much less than those in plasma and its reported absence from C.S.F.

Glucose reacts rapidly with alkaline picrate at high temperatures but at 20°C. it reacts only slowly, giving a colour which increases with time for several hours. An increase

of colour after 20 min. has been reported to occur when alkaline picrate is added to creatinine solution containing glucose (Hunter and Campbell, 1917; Lauson, 1951) or to glucolysed plasma or urine (Haugen, 1953). A solution of glucose containing 100 mg./100 ml. was reported by Hunter and Campbell to give, after 10 min., the same colour density as that given by a creatinine solution containing 0.03 mg./100 ml. Lauson reported that a similar glucose solution gave, after 20 min., the colour density given by a creatinine solution containing 0.02 mg./100 ml. Roettger (1953) reported that glucose added to creatinine solutions produced an increase in colour density somewhat more than the above figures. Glucose was found to be destroyed by the NC-bacteria although not to be adsorbed by Lloyd's reagent.

From these findings, however, it appears that glucose in plasma or serum filtrates could only form a small fraction of the non-creatinine chromogen.

Other substances which have been suggested as possible interfering substances are pyruvic acid, acetoacetic acid and acetone.

Kostir and Co-workers (1949, 1951) claimed to have demonstrated pyruvic acid as an interfering substance in serum filtrates by the use of chromatography and that it accounts for half the total chromogen present. They proposed the treatment of filtrates with ceric sulphate to destroy the pyruvic acid before applying the Jaffe reaction.

It was confirmed that pyruvic acid is a chromogen but the colour obtained was much less than that reported by Kostir and Rabek (1949), although it did increase for several hours. Pyruvic acid was found to be destroyed by the NC-bacteria but it was not adsorbed on Lloyd's reagent. The ceric sulphate method was examined but no significant difference was found in the chromogen contents of the filtrates before and after treatment with ceric sulphate. However, the amount of pyruvic acid which is believed to be present in plasma (Lu, 1939; Bueding and Wortis, 1939) could only account for a small fraction of the non-creatinine chromogen and, furthermore, it is generally agreed that in the absence of enzyme inhibitors pyruvic acid disappears very rapidly from blood after withdrawal from the body, so that even in the short time required for clotting to take place, it has largely been destroyed.

The colour given by acetoacetic acid is such that the amount believed to be present in plasma could only account for a fraction of the non-creatinine chromogen. Acetoacetic acid was, however, found to be destroyed by the NC-bacteria although it was not adsorbed on Lloyd's reagent.

Acetone gives a colour which increases for several hours but the amount believed to be present in plasma is likewise only sufficient to account for a very small fraction of the non-creatinine chromogen. It was found not to be destroyed by the NC-bacteria nor to be adsorbed on Lloyd's reagent.

Creatinine Determinations with Other Reagents.

1. 3:5 dinitro-benzoic acid.

The use of this reagent for the determination of creatinine was suggested independently from three different laboratories (Behre and Benedict, 1936; Bollinger 1936; Langley and Evans, 1936) and it has been claimed that it provides a more specific reaction for the determination of creatinine than the Jaffe reaction (Langley and Evans, 1936; Miller and Dubos, 1937a; Roettger, 1949). In general, however, the reaction has been reported to be less satisfactory than the Jaffe reaction. Difficulties have involved fading of the colour, pH adjustment and purification of the reagent (Andes, 1938; Fister, 1950; Roettger, 1949).

Using this reagent with "neutral" tungstic acid filtrates Miller and Dubos (1937b) found that creatinine contents of serum were 99% (94-106%) of those obtained with the Jaffe reaction (both values uncorrected for non-creatinine chromogen). Subtracting 10%, the mean value obtained by Miller and Dubos, from the total chromogen content determined by the Jaffe reaction to allow for the non-creatinine chromogen, the total chromogen determined by the dinitrobenzoic acid method becomes on the average 9% higher than the corrected picric acid figures. Langley and Evans (1936) reported that the mean value for total chromogen in serum using the dinitrobenzoic acid method was 2% higher than that obtained using the Jaffe reaction. Mandel and

Jones (1953) reported that with trichloracetic acid filtrates the dinitrobenzoic acid method gave values for serum creatinine considerably (30-40%) higher than those obtained with Lloyd's reagent method using picric acid. Applied to the eluate from Lloyd's reagent the results with dinitrobenzoic acid were still slightly higher than those obtained with picric acid. In a small series Scandrett (1952), using the NC-bacteria, found that the non-creatinine chromogen contents of filtrates determined with dinitrobenzoic acid were the same as those obtained using picric acid.

It appears, therefore, that the value for the total chromogen in serum using dinitrobenzoic acid is higher than the creatinine content obtained using either the NC-bacteria or Lloyd's reagent. This suggests that there is a fraction of the total chromogen determined with dinitrobenzoic acid which corresponds to the non-creatinine chromogen determined by the picric acid method and that the reaction is therefore no more specific than the Jaffe reaction.

2. Method of Barclay and Kenney (1947).

This is a nephelometric method based on the reaction between creatinine and a reagent prepared from Nessler's reagent. The reaction has been claimed to be more specific for creatinine than the Jaffe reaction, but no comparative figures for creatinine in serum and urine determined by the Jaffe reaction and by nephelometric method are given. However, comparative

results for the creatinine contents of other tissues are given and these suggest that the specificity of this method towards creatinine is less than that obtained using the NC-bacteria.

Conclusions and Summary.

Examination of the Jaffe reaction has shown that the conditions under which the reaction is carried out must be carefully controlled with regard to the time and temperature of the colour development and to the acidity of the solution containing creatinine. Using the method of Folin and Wu for the colour development the optical density of the colour obtained by development for 20 min. at 20°C. and measured at a wavelength of 520 mu is directly proportional to the amount of creatinine present up to a concentration of 400 ug/100 ml.

The optical densities of alkaline picrate and of alkaline creatinine picrate are dependent on the pH of the final solution, which, in turn, depends on the acidity of the original solution. Since the acidities of serum filtrates vary according to the method of preparation, determination of "creatinine" as total chromogen requires that the spectrophotometer should be calibrated with standard creatinine solutions of similar acidity or, alternatively, that the filtrates should be neutralised. The latter procedure is the more practicable.

The recovery of creatinine added to plasma or serum is influenced by the pH of the protein-free filtrate and is minimal (85-90%) with filtrates having a pH of about 3.5. When the

filtrate pH is below 2.0 the recovery is quantitative. When allowance is made for the effect of the acidity of the filtrate on the optical densities the recovery of creatinine added to filtrates is virtually 100%. Recoveries from urine are quantitative. It is concluded that the failure to obtain a quantitative recovery of creatinine added to plasma or serum using some methods of preparing protein-free filtrates is due to the adsorption of creatinine by the precipitated protein.

When the Jaffe reaction is applied to the determination of creatinine in plasma, serum or urine allowance must be made for the presence of non-creatinine chromogen. Using the NC-bacteria or Lloyd's reagent the "creatinine" content of serum filtrates, or of diluted urines, is lower than the total chromogen content by a statistically significant amount. However, none of the "specific" methods which have been proposed are entirely satisfactory.

The adsorption method using Lloyd's reagent in addition to improving the specificity eliminates any effect of the filtrate acidity, and the possible effect of other filtrate constituents, on the optical densities of alkaline picrate and alkaline creatinine picrate. However, the results of the experiments carried out with dialysed plasma or serum suggest that a small fraction of the non-creatinine chromogen present in filtrates can be adsorbed on Lloyd's reagent.

The use of the NC-bacteria improves the specificity to an approximately similar extent, but the NC-bacteria can destroy

possible interfering chromogens, e.g. pyruvic acid. Moreover, since the non-creatinine chromogen is reported to have an inhibiting effect on the colour development it may not be correct to subtract the non-creatinine chromogen content of filtrates determined with the NC-bacteria from the total chromogen content to obtain the true creatinine content.

The use of the dinitrobenzoic acid method or of the nephelometric method do not appear to offer any advantages over the use of the NC-bacteria or Lloyd's reagent.

It is concluded that the use of Lloyd's reagent with "acid" filtrates of plasma or serum, or with diluted urine, provides, at present, the most satisfactory method for the determination of the creatinine content of these fluids.

III. THE EXCRETION OF CREATININE IN MAN.

The Excretion of Creatinine in Man.

The mechanism of the renal excretion of creatinine in man has been examined by many investigators who have used methods involving three principles.

1. The examination of the relationship between the plasma concentration and the rate of excretion of creatinine.

2. The comparison of simultaneously determined clearances of creatinine and inulin.

3. The examination of the effect on creatinine clearance of substances known to influence renal tubular activity.

It is convenient to deal separately with each of these aspects of the subject.

The Relationship between the Plasma Concentration and the Rate of Excretion of Creatinine.

The rationale for the examination of the relationship between the plasma concentration of a substance and the rate of its excretion as a method of obtaining information about the mechanism of its excretion has already been discussed in detail.

The problems which have been encountered in investigating the excretion of creatinine have by no means been entirely physiological and difficulties in the accurate determination of creatinine in plasma, serum or urine, especially at endogenous levels, have confronted every worker in the field and have no doubt contributed to the existing confusion. It is important, therefore, (in interpreting the results of clearance determinations) to take in to account the analytical method which has been

used by each worker, considering in particular the specificity of the method.

In 1926, Rehberg, finding that creatinine was concentrated in urine more than any other substance normally present in plasma and believing that tubular excretion of substances did not normally occur suggested that the creatinine clearance might form a measure of the glomerular filtration rate. He did not, however, examine critically the relationship between UV and P.

Mackay (1929-30) examined this relationship in one individual over a range of plasma concentrations from 5 - 15 mg./100 ml. and found that the rate of excretion was proportional to the plasma concentration.

Cope (1931) also examined this relationship in one individual but found that the relationship was only one of direct proportionality if 0.5 mg./100 ml. was subtracted from the determined plasma concentration as an arbitrary correction for the non-creatinine chromogen.

Jolliffe and Smith (1933) examined this relationship in two more individuals using a specific method involving the adsorption on kaolin (Shannon, Jolliffe and Smith, 1932), for the determination of creatinine at low plasma levels. They found that the rate of excretion was directly proportional to the plasma concentration in both individuals up to plasma concentrations of 7.0 and 11.6 mg./100 ml. respectively.

Shannon (1935) examined this relationship in four normal persons at higher plasma concentrations (P_{cr} 10-100 mg./100 ml.) following single intravenous injections of creatinine. He determined inulin clearances simultaneously and expressed his results

in terms of creatinine/inulin clearance ratios as well as in terms of the creatinine clearance. He found that as the plasma creatinine concentration was increased from 10 mg./100 ml. to 100 mg./100 ml. the clearance ratio decreased from 1.4 to 1.1 as a result of an absolute decrease in the creatinine clearance since the inulin clearance remained virtually constant throughout the experiment. A summary of Shannon's data is given in Table 30 which also contains summaries of the relevant data of other investigators.

Shannon concluded that the depression of the clearance of creatinine, both in absolute value and relative to the inulin clearance, was evidence strongly suggestive of the excretion of creatinine by the renal tubules.

One of the features of this investigation which must be considered further was the anomalous finding that as plasma creatinine concentration decreased from the highest level the creatinine/inulin clearance ratio remained constant at values of 1.1 to 1.2 for some hours. Shannon was unable to offer a satisfactory explanation for this though he states - "It seems probable, therefore, that our creatinine inulin clearance ratios as recorded in these figures (Fig. I and II) are mostly too low, being "set" or determined by a plasma level that was initially higher than actually observed." The significance of this comment is not clear. In view of the possible influence of delay-time effects it seems more likely that the clearances determined soon after the injection of creatinine are erroneously high and that the ratios obtained some time after the injection, are more correct, since it appears that the effect of delay-time is

TABLE 30. Creatinine Clearance and Plasma Concentration in Normal Subjects - Summary of Literature.

Author	N	Creatinine Administration	Pcr	Reference Substance & Administration	Ccr & Ccr./Cin.
			mg./100ml.		
Ma cKay (1929-30)	1	Oral	5 - 15	-	Ccr. independent of P.
Cope (1931)	1	Oral	End. - 8	-	Ccr. dependent on P (UV = k(P - 0.5)
Jolliffe & Smith (1933)	2	Oral	End. - 7 End - 12	-	Ccr. independent of P.
Shannon (1935)	4	Oral & inj.	10 40-100	Inulin inj.	Ccr. falls when P is increased. Ccr. constant when P decreased. Ccr/Cin behaved similarly.
Findley (1937)	4	Oral	End. - 10	-	Ccr. dependent on P (UV = k(P - 0.5)
Winkler & Para (1937)	8 6	Oral & injection	4-17 4-20	Sucrose inj.	Ccr. & Ccr/Cin. tended to fall as P decreased.
Miller & Winkler (1938)	5	infusion	End., 10-20	Inulin infusion.	Ccr. & Ccr/Cin. increased at high level and then fell.
Shannon & Ranges (1941)	6	Oral	6-12	Inulin infusion.	Ccr. & Ccr./Cin. fell as P decreased. Temporarily raised by injection.
Steinitz & Turkand (1941)	22	Oral	End. 5-12	Inulin infusion	Ccr. & Ccr/Cin. increased at higher P
Brod & Sirota (1948)	4	infusion	End. 80-100	Mannitol infusion.	Ccr. increased at high level of P.

TABLE 30 (Contd).

Author	N	Creatinine Administration	Pcr	Reference Substance & Administration	Ccr. & Ccr./Cin.
Hare (1950)	2 7	infusion	-	Inulin? infusion	Ccr. very slightly increased at higher levels.
Mandel et al.	3	Oral	-	Inulin	Ccr. markedly raised at high levels of P in 2 out of 3 cases.

insignificant 60 min. after injection. Insufficient data regarding the creatinine clearances at any one level are given to test this hypothesis. In spite of these anomalies, however, Shannon's conclusions regarding the excretion of creatinine by the renal tubules have been generally accepted up to the present time.

Findley (1937) re-examined the relationship between the rate of excretion of creatinine and its plasma concentration at low levels (P_{cr} 10 mg./100 ml.) in 4 normal subjects who had been given creatinine orally. He found, in agreement with Cope, that, if 0.5 mg./100 ml. was deducted from the observed plasma concentration as an arbitrary correction for the non-creatinine chromogen, the rate of excretion was proportional to the plasma concentration over the range from 1.0 to 14 mg./100 ml. and argued that the high plasma levels employed by Shannon were outwith the physiological range and that clearance determinations at these levels were therefore unreliable.

Winkler and Parra (1937) also examined the excretion of creatinine at low plasma levels (P_{cr} 20 mg./100 ml.), administering the creatinine either orally or by single injection, but making no correction for delay-time. In more than half the cases the creatinine clearance decreased as the plasma concentration decreased. In some cases simultaneous sucrose clearances were determined following a single injection of sucrose. After oral creatinine the creatinine/sucrose clearance ratios in two out of four cases (2/4) decreased, while in the other two cases it remained constant. After I.V.I. of creatinine in 3/4 cases the ratio decreased while in 1/4 cases the ratio increased. The mean initial ratio was 1.51 after oral administration and

1.63 after I.V. administration; the final ratios were 1.25 in both series.

The data of Winkler and Parra can be summarised by stating that the creatinine clearance, both in absolute value and relative to the sucrose clearance, tended to decrease during the course of the experiment. In general the interpretation of these data is made difficult by the variability of the sucrose clearances, and to a smaller extent of the creatinine clearance in individual cases and to the fact that all the clearances were determined with decreasing plasma concentrations. Nevertheless the authors concluded that the decreasing creatinine clearances noted by themselves and those originally noted by Shannon and attributed to saturation of the tubular excretion of creatinine at high levels were in fact the same phenomenon and thus could not be related solely to changes in plasma concentration since the latter had been in opposite directions in the two investigations.

Miller and Winkler (1938) compared creatinine/inulin clearance ratios at endogenous plasma creatinine levels with those at moderately high plasma creatinine levels (P_{cr} = 20 mg./100 ml.) obtained by the continuous infusion of creatinine using the NC-bacteria for the determination of creatinine at the endogenous levels. They claimed that the mean exogenous/endogenous creatinine clearance ratio was 1.9, using, apparently the values of the last collection period at the endogenous level and of the first period at the exogenous level for this calculation. Since the creatinine clearances in any one individual were variable calculation of the ratio of the average exogenous creatinine/

inulin clearance ratio to the average endogenous creatinine clearance ratio would appear to be a more accurate method of deriving this figure. When this is done a mean ratio of 1.4 is obtained.

Steinitz and Turkand (1940) examined the excretion of creatinine after oral administration and reported a mean exogenous/endogenous clearance ratio of 1.2 (0.9 - 1.9).

Shannon and Ranges (1941) examined this problem further administering creatinine orally and by infusion and comparing creatinine clearances with simultaneous inulin clearances. They confirmed the fall in creatinine/inulin clearance ratio after a single injection which had been noted by Winkler and Parra but reported that this fall was not present when creatinine was given by a continuous infusion.

After oral administration the mean initial creatinine inulin clearance ratio was 1.45 (P_{cr} = 10 mg./100 ml.) and this decreased to 1.15 (P_{cr} = 5-7 mg./100 ml.) after five hours. At this stage an injection of creatinine was found to elevate the ratio to a value of between 1.25 and 1.55 but immediately after the injection the ratio tended to decrease again. Since collections were made within a short time of the injection (20-40 min.) it would seem likely that the elevation of the depressed ratio was simply an effect of the method of giving the second dose of creatinine, i.e. a delay-time effect. This is borne out by the fact that after the second dose of creatinine the ratios tended to fall much more rapidly than after the initial dose.

Three possible explanations of this phenomenon were considered

by these authors

1. Fatigue of the excretion mechanism.

2. Temporarily increased excreting activity following an increase in plasma concentration. (Cf. data of Miller and Winkler, 1938).

3. Conversion in the body of the administered creatinine to an unknown chromogen which was excreted by the kidney by a mechanism different from that of the excretion of creatinine.

They argued that since a depression of the ratio was not obtained when a continuous infusion of creatinine was given fatigue of the renal excreting mechanism were unlikely the first two explanations. They concluded that conversion of creatinine to an unknown chromogen was the most likely explanation although they were unable to obtain any direct evidence for such a conversion.

Brod and Sirota (1948), using continuous infusions of creatinine and inulin obtained a mean exogenous/endogenous ratio of 1.61 (1.33-1.87) in four normal subjects. Exogenous creatinine concentrations varied from 80 - 100 mg./100 ml. No data regarding the timing of the injections and of the collection periods is available.

Hare (1950), using Lloyd's reagent for the determination of the creatinine concentration at endogenous levels reported that the creatinine/inulin clearance ratio was 1.03 at endogenous levels and 1.08 at exogenous levels. The exogenous concentrations were not, however, stated and this series contained all age groups.

Mandel et al. (1953) reported that oral administration of

creatinine raised the creatinine/inulin clearance ratio from 0.90 to 1.74 in one subject but had no effect in a second subject.

The above findings have concerned normal subjects. In subjects with renal disease the relationship between the rate of excretion and the plasma concentration has been examined by three groups of workers.

Winkler and Parra (1937) using sucrose as a reference substance and administering creatinine by mouth and by single injection obtained results similar to those obtained with normal subjects, i.e. a tendency for the creatinine/sucrose clearance ratio to decrease with decrease in creatinine plasma concentration.

Miller and Winkler (1938) using continuous infusions of creatinine and the NC-bacteria for the determination of endogenous plasma concentrations obtained a mean exogenous/endogenous clearance ratio of 1.13 (0.76-1.45) in four subjects with renal disease.

Brod and Sirota (1948) also used infusions of creatinine and obtained exogenous/endogenous clearance ratios of 1.15 and 1.23 in two subjects with renal disease. The exogenous levels were 40 and 80 mg./100 ml. respectively.

The above data as far as normal subjects are concerned are summarised in Table 30, from which it will be seen that there is no consistent pattern in the results which have been obtained by different workers, using in general, different technical methods. The majority of investigators have concluded that in normal subjects creatinine tolerance is dependent on the plasma concentration but do not agree on the nature of this dependence. In the investigations on subjects with renal disease the authors of all three

groups agree in concluding that creatinine clearance is dependent on the plasma concentration.

In view of the errors which may be involved in determining clearances when plasma concentration is not constant it seems valid to exclude from further discussion data obtained while the plasma concentration was not constant. As far as normal subjects are concerned this leaves only the data of Miller and Winkler since the method of creatinine administration and the exogenous level used by Hare (1950) are not available from her data.

In view of the importance of establishing the mechanism by which creatinine is excreted by the human kidney it was considered desirable to examine further the relationship between the rate of excretion of creatinine and the plasma concentration by infusion experiments using inulin as a reference substance. The initial experiments were carried out using different rates of infusion to obtain different levels of creatinine and inulin. In the later experiments concentrated solutions of creatinine and inulin were infused by means of a constant rate infusion pump at a comparatively slow rate to avoid the possible effect of the infusion of large volumes of saline (Crawford and Ludeman, 1951; Crawford and Gaudino, 1952).

Experimental.

The rate of excretion of creatinine was measured at the endogenous plasma creatinine level and at two higher levels in nine experiments involving seven subjects. Of these subjects, five were normal healthy individuals; the other two had mild

diastolic hypertension but were otherwise perfectly normal. Simultaneous inulin clearances were also determined, the plasma inulin concentration being maintained at one level in five experiments and two or three different levels in the others.

The experiments were carried out with the subjects in the post-absorptive state and, to ensure a good urine flow, about 500 ml. of water were given to the subjects at the start of the experiment and, during the experiment, water was given at the rate of 200 ml. every 30 min.

The creatinine administered was supplied by L. Light & Co. It was prepared for use by being dissolved in sterile pyrogen-free 0.9% (w/v) sodium chloride solution at 90 - 100°C. and filtered through an autoclaved Seitz filter. The inulin was prepared according to the method previously described.

Plasma concentrations of creatinine and inulin were established and maintained by appropriate priming injections and sustaining infusions given into a vein in the forearm. A constant speed infusion pump delivering 0.60 ml./min. was employed and the different plasma levels were maintained by altering the concentration of the infusate.

Clearance measurements were started 30 min. after the start of the sustaining infusion and clearance periods were 10-40 min. in duration. Blood was obtained by venepuncture at the start of the experiment for the inulinoid blank and at the beginning and end of each period using the contralateral arm.

Serum was used for the determination of creatinine and inulin. In all experiments except three, urine was collected by

means of an indwelling soft rubber catheter and at the end of each period of urine collection the bladder was washed out with 20 ml. of sterile saline followed by insuflation and expression of air. In three experiments urine was spontaneously voided and this gave satisfactory collections in all but a few periods.

Creatinine in serum and urine was determined by the method previously described. In the determinations in serum "neutral" tungstic acid filtrates were used* and the NC-bacteria were employed to obtain the true creatinine concentrations in serum and urine at the endogenous level. Plasma concentrations at higher levels were corrected for the non-creatinine chromogen. Inulin in serum and urine was determined by the method previously described.

Clearances were calculated by dividing the average minute rate of excretion by the mean of the plasma concentrations at the beginning and at the end of the collection periods. No correction was made for delay-time since the plasma concentrations at any one level were virtually constant.

Results.

The detailed results of these experiments are given in Tables 31-39.

The results have been analysed in three ways:-

* The fact that "acid" tungstic acid filtrates give higher values for the creatinine content of serum and that these higher values are probably more accurate was not appreciated until most of these experiments had been carried out.

TABLE 31. Creatinine Infusion Experiment No. 1. Subject W.H. (1).

Time	Procedure	Period Duration	Urine flow	Plasma creatinine	Cr. excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin.
min.		min.	ml./min	mg./100 ml	mg./min.	ml./min.	mg./100 ml	mg./min	ml./min	
0	P.* 1.5 g. Inulin									
28-37	S. 40 mg./min. Inulin	9.0	7.3	0.87	1.16	133	22.0	20.3	93	1.43
-48	in 2 ml. Saline	11.3	6.0		1.09	133	21.8	17.3	80	1.67
-60		12.3	6.9		1.20	138	22.1	21.2	96	1.43
-75		14.0	7.0		1.15	132	22.8	17.7	77	1.69
85	P. 6 g. Creatinine									
	S. 40 mg./min. Inulin									
121-133	6 mg./min Creatin-	12.0	7.9	3.70	5.2	140	22.6	18.6	83	1.67
-146	ine in 3 ml. Saline	13.0	5.7	3.63	4.8	132	22.6	18.4	82	1.61
-161		14.8	4.9	3.78	4.8	127	22.8	17.1	75	1.69
165	P. 6 g. Creatinine									
	S. 40 mg./min. Inulin									
195-207	12 mg./min. Creat-	11.5	5.0	7.43	9.5	128	28.0	19.7	74	1.72
-222	inine in 3.5 ml.	15.5	6.7	7.60	10.5	138	31.7	25.4	81	1.69
-230	Saline	8.0	8.5	7.70	11.1	144	31.0	31.0	100	1.43
-239		9.0	8.9	8.00	11.3	140	35.5	31.7	89	1.56
240	P. 6 g. Creatinine									
	S. 40 mg./min. Inulin									
270-282	24 mg./min. Creatin-	12.5	8.5	12.5	15.7	126	34.5	29.8	79	1.59
-292	ine in 5.0 ml.	10.0	10.1	12.8	18.5	144	34.0	34.9	103	1.39
-302	Saline	9.5	9.4	13.1	17.3	133	34.7	32.9	95	1.41
-312		10.0	8.7	13.1	18.6	140	36.5	34.6	95	1.47

* P = Priming injection.

S = Sustaining infusion.

TABLE 32. Creatinine Infusion Experiment No. 2. Subject W.H.(2)

Time	Procedure	Period Duration	Urine flow	Plasma Creatinine	Cr. Excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin.
min.		min.	ml./min.	mg./100ml	mg./min	ml./min	mg./100ml	mg./min	ml/min.	
0	P. 2.5 g. Inulin									
	S. 25 mg./min. Inulin									
41-55	in 1 ml. Saline	14.5	2.0	0.87	0.99	114	30.3	28.8	95	1.20
-67		12.3	3.1		0.94	108	28.4	27.5	96	1.12
-38		11.0	3.7		1.03	119	27.6	28.6	103	1.16
-89		10.8	3.7		0.99	107	25.3	25.7	101	1.06
89	P. 2.5 g. Inulin									
	2.5 g. Creatinine									
	S. 50 mg./min. Inulin									
126-138	15 mg./min. Creatin-	11.5	7.3	9.3	12.2	131	52.8	55.0	102	1.28
-153	ine in 3 ml. Saline	15.3	8.6	8.5	11.8	138	54.1	59.1	101	1.34
-164		10.8	7.7	8.1	11.1	134	54.1	50.2	93	1.45
-176		12.8	9.4	8.0	11.7	146	54.0	53.7	100	1.45
177	P. 2.5 g. Inulin									
	15 g. Creatinine									
	S. 75 mg./min. Inulin									
211-220	75 mg./min. Creatin-	8.5	16.7	63.1	79.0	125	80.8	77.3	96	1.37
-228	ine in 5 ml. Saline	8.8	16.0	64.1	55.5	118	83.0	78.9	95	1.25
-241		12.5	14.2	59.6	69.1	115	83.5	76.7	92	1.25
-250		9.5	15.1	58.1	72.4	125	84.5	83.3	99	1.27
251	P. 3.5 g. Inulin									
	15 g. Creatinine									
	S. 100 mg./min. Inulin									
278-300	150 mg./min. Creatin-	22.0	14.0	118	134	113	139	123	89	1.33
-309	ine in 9 ml. Saline	9.0	11.9	115	133	115	143	143	100	1.15
-319		9.3	8.0	112.5	(96)	(88)	142	(105)	(77)	1.15
-329		11.0	9.9	110	130	118	138	125	91	1.30

TABLE 33. Creatinine Infusion Experiment No. 3. Subject W.H.(3)

Time		Procedure	Period Duration	Urine flow	Plasma Creatinine	Cr. Excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin.
min.			min.	ml./min.	mg./100 ml.	mg./min.	ml./min.	mg./100 ml.	mg./min.	ml./min.	
0	P.	1.5 g. Inulin									
	S.	30 mg./min. Inulin									
34-56		in 0.6 ml. Saline	22.3	1.0	0.95	0.81	86	27.6	14.4	60	1.57
-75			18.5	1.6		1.21	127	26.7	22.7	85	1.60
-94			18.8	2.3		1.07	113	25.1	20.2	81	1.47
97	P.	5.0 g. Creatinine									
	S.	30 mg./min. Inulin,									
		18 mg./min. Creatin-									
128-145		ine in 0.6 ml. Saline	16.5	9.0	13.2	17.0	130	28.3	26.4	93	1.40
-165			20.0	6.5	13.2	15.9	121	28.4	22.0	80	1.51
169	P.	20 g. Creatinine									
	S.	30 mg./min. Inulin;									
		60 mg./min. Creatin-									
220-242		ine in 0.6 ml. Saline	21.5	5.0	68.4	83.9	123	27.0	28.2	104	1.18
-263			21.5	3.1	64.0	66.1	103	27.7	27.2	98	1.03
-280			17.0	2.8	59.0	61.1	103	28.0	22.4	80	1.29

TABLE 34. Creatinine Infusion Experiment No. 4. Subject W.R.

Time	Procedure	Period Duration	Urine Flow	Plasma creatinine	Cr Excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin
min.		min.	ml./min.	mg./100ml	mg./min.	ml./min.	mg./100ml	mg./min	ml./min.	
0	P. 0.6 g. Inulin									
	S. 5 mg./min. Inulin									
38-50	in 2 ml. Saline	12.3	2.0		1.09	148	5.0	6.5	128	1.56
-63		12.5	3.5		1.12	152	4.7	6.2	132	1.61
-75		15.5	3.6	0.74	1.13	153	5.2	6.0	114	1.67
-84		9.0	13.4		1.11	150	5.3	6.2	109	1.47
-93		9.0	16.8		1.17	158	5.0	6.8	134	1.41
	P. 1.8 g. Inulin									
	2.5 g. Creatinine									
	S. 30 mg./min. Inulin									
123-133	15 mg./min. Creatinine	9.8	10.8	4.8	7.7	160	18.0	22	121	1.32
-147	in 2 ml. Saline	13.5	8.6	4.8	7.6	158	21.2	22.5	106	1.49
-159		11.8	8.2	4.8	8.1	167	22.4	26.1	114	1.47
-170		11.3	6.2	4.8	7.6	157	21.1	22.2	105	1.49
	P. 3.0 mg. Inulin									
	15 g. Creatinine									
	S. 75 mg./min. Inulin									
210-222	75 mg./min. Creatinine	12.3	5.0	50.4	77.5	144	61.2	73.5	120	1.20
-235	in 4.5 ml. Saline	13.0	4.5	46.3	79.0	169	58.7	71.1	121	1.39
-248		13.0	4.0	44.2	71.6	160	56.2	63.7	113	1.43
-260		12.0	3.3	44.4	65.6	147	55.2	61.5	112	1.32
	P. 6.0 g. Inulin									
	15 g. Creatinine									
	S. 150 mg./min. Inulin									
290-302	150 mg./min. Creatinine	11.5	8.9	80.0	129.0	161	123.1	166.0	137	1.17
-313		11.0	10.0	86.0	133.5	154	118.2	155.4	132	1.16
-315		11.5	10.3	87.8	147.0	167	114.2	158.6	138	1.20
-321		6.0	8.8	86.3	127.6	148	112.2	134	120	1.23

P. = Priming injection
S. = Sustaining infusion.

TABLE 35. Creatinine Infusion Experiment No. 5. Subject J.C.

Time		Procedure	Period Duration	Urine Flow	Plasma Creatinine	Cr. Excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin.
min.			min.	ml./min	mg./100ml	mg./min.	ml./min.	mg./100ml	mg./min	ml./min.	
0	P.	0.6 g. Inulin									
	S.	5.0 mg./min.									
30-47		in 2.0 ml. Saline	17.0	3.5	0.59	0.84	143	12.0	12.8	107	1.33
-60			13.0	5.0	0.59	0.87	148	9.8	10.6	108	1.37
-73			13.0	6.4	0.99	0.86	146	9.1	9.9	109	1.33
-86			12.5	5.5	0.59	0.86	146	8.4	9.2	109	1.33
86	P.	1.8 g. Inulin									
		2.5 g. Creatinine									
	S.	30 mg./min. Inulin									
96-106		15mg./min. Creatinine	9.5	10.1	8.6	13.1	153	28.7	34.1	119	1.28
-120		in 2 ml. Saline	14.5	4.1	8.8	12.5	143	31.1	32.4	105	1.35
-138			18.0	4.3	8.9	13.1	147	34.2	34.6	101	1.45
-151			12.5	7.2	8.9	15.5	174	36.2	43.1	119	1.47
152	P.	3.0 g. Inulin									
		15 g. Creatinine									
	S.	75 mg./min. Inulin									
192-202		75 mg./min. Creatinine	10.0	9.5	51.8	73.4	141	77.5	82.1	106	1.33
-216		in 5 ml. Saline	14.0	4.7	46.4	60.3	140	66.0	72.6	110	1.16
-218			11.5	3.6	44.4	62.4	141	61.7	65.9	107	1.32
-232			14.0	3.4	45.5	65.5	145	61.7	63.5	103	1.41
235	P.	6.0 g. Inulin									
		15 g. Creatinine									
	S.	150 mg./min. Inulin									
269-280		150 mg./min. Creatinine	11.0	12.8	105.2	189	156	171	193	113	1.27
-289		in 9 ml. Saline	9.0	12.9	116.2	174	149	162	210	129	1.19
-296			6.5	14.7	122.4	209	170	156	245	157	1.15
-306			10.0	12.4	224.9	190	152	176	211	120	1.27

P. = Priming injection.
S. = Sustaining Infusion.

TABLE 36 Creatinine Infusion Experiment 6. Subject M.

Time		Procedure	Period Duration	Urine flow	Plasma creatinine	Cr. excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	CCr/Cin.
min.			min.	ml./min	mg./100 ml	mg./min	ml./min	mg/100 ml	mg./min.	ml./min	
0	P.	2.5 g. Inulin									
	S.	30 mg./min.									
62-90		Inulin	27.5	13.4	0.92	1.27	138	28.4	26.4	93	1.48
-115			25.0	15.7		1.24	134	29.6	27.5	93	1.47
133	P.	5.0 g. Creatinine									
	S.	30 mg./min. Inulin;									
		18 mg./min. creatinine									
189-216			27.0	10.9	13.8	19.2	139	36.5	29.2	80	1.74
220	P.	20 g. Creatinine									
	S.	30 mg./min. Inulin;									
		60 mg./min. Creatinine									
254-293			39.0	6.3	60.1	72.9	121	30.0	26.7	89	1.36

TABLE 37. Creatinine Infusion Experiment No. 7. Subject P.

Time	Procedure	Period Duration	Urine flow	Plasma creatinine	Cr. excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin.
min.		min.	ml./min.	mg./100 ml	mg./min.	ml./min.	mg/100 ml	mg./min.	ml./min.	
0	P. 2.5 g. Inulin S. 30 mg./min.									
21-41	Inulin	20.3	18.5	0.75	1.54	206	25.6	29.5	115	1.79
-57		15.3	22.6		1.44	194	24.7	28.2	114	1.71
80	P. 5.0 g. Creatinine S. 30 mg./min. Inulin 18 mg./min. Creatinine in 0.6 ml. Saline									
125-140		14.5	15.2	11.7	18.5	(157	23.5	29.2	124	1.27
-155		12.5	12.0	11.2	17.3	153	22.8	26.0	114	1.34
160	P. 20 g. Creatinine S. 60 mg./min. Creatinine; 30 mg./min.									
196-215	Inulin	18.5	6.3	40.1	55.9	150	33.7	39.5	118	1.27
-251		36.5	2.7	35.7	43.3	123	29.8	31.3	105	1.17

P = Priming injection

S = Sustaining infusion.

TABLE 38 Creatinine Infusion Experiment No. 8. Subject W.

Time		Procedure	Period Duration	Urine flow	Plasma creatinine	Cr. excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin
min.			min.	ml./min	mg/100 ml	mg./min	ml./min.	mg./100 ml	mg./min	ml./min	
0	P.	2.5 g. Inulin									
	S.	30 mg./min. Inulin in									
47-72		0.6 ml. Saline	23.5	4.3	0.75	1.33	178	23.1	26.6	115	1.55
-94			21.5	12.0		1.28	171	22.8	25.8	113	1.51
103	P.	5.0 g. Creatinine									
	S.	30 g./min. Inulin; 18 g./min. Creatinine in 0.6 ml.									
153-168		Saline	16.0	16.3	11.5	20.0	173	21.0	25.5	121	1.43
180	P.	10 g. Creatinine									
	S.	30 g./min. Inulin 60 g./min. Creatinine in 0.6 ml.									
215-240		Saline	24.5	8.8	34.0	54.4	160	22.6	24.8	109	1.47
-260			20.0	13.3	32.2	52.7	163	22.6	25.8	114	1.43

TABLE 39. Creatinine Infusion Experiment No. 9 Subject C.M.

Time		Procedure	Period Duration	Urine flow	Plasma Creatinine	Cr. Excreted	Creatinine Clearance	Plasma Inulin	Inulin Excreted	Inulin Clearance	Ccr/Cin.
min.			min.	ml./min	mg./100 ml	mg./min	ml./min.	mg./100 ml.	mg./min.	ml./min.	
0	P.	3.0 g. Inulin									
	S.	20 mg./min. in									
7-26		1 ml. Saline	19.3	2.0		0.945	105				
-45			18.8	1.3	0.90	0.786	87				
-60			14.3	2.8		1.04	116				
-73			13.3	3.7		0.838	94	30.4	24.0	72	1.30
84	P.	40 g. Creatinine									
115	P.	4.5 g. Inulin									
	S.	50 g./min. Creatin-									
154-168		ine in ml. Saline	14.3	6.1	99.4	105.1	95			80	1.19
-181			12.8		93.8	87.0	93			76	1.22
-199			14.0		89.8	77.4	86			67	1.28
-222			26.5		84.61	78.6	93			75	1.23

TABLE 40. Creatinine Clearance (Ccr) and Plasma Creatinine Concentration (Pcr).

Subject	Pcr ≒ 1			Pcr ≒ 10			Pcr ≒ 50			Pcr ≒ 100		
	Ccr Ml/min	% Ccr Pcr = 1	% Ccr Pcr = 10	Ccr Ml/min	% Ccr Pcr = 1	% Ccr Pcr = 10	Ccr Ml/min	% Ccr $P_{cr.}$ = 1	% Ccr $P_{cr.}$ = 10	Ccr Ml/min	% Ccr $P_{cr.}$ = 1	% Ccr $P_{cr.}$ = 10
W.H.(1)	134	100	98	137	102	100	-	-	-	-	-	-
W.H.(2)	110	100	80	137	125	100	121	110	88	115	104	84
W.H.(3)	116	100	92	126	109	100	110	96	87	-	-	-
W.R.	152	100	95	161	105	100	152	102	96	158	104	98
J.C.	146	100	94	154	106	100	142	97	92	154	106	100
C.M.	100	100	-	-	-	-	-	-	-	92	92	-
M.	136	100	98	139	102	100	121	89	87	-	-	-
P.	200	100	132	155	78	100	137	68	88	-	-	-
W.	175	100	101	173	79	100	162	92	93	-	-	-
Mean	141	100	99	148	103	100	135	93	90	130	102	94
P*	-	-	0.7	-	0.3	-	-	0.4	0.05	-	-	-

*P = probability of difference from 100 being due to chance alone.

1. Comparison of creatinine clearances determined at different plasma creatinine levels.

2. Regression analysis of the relationship between the rate of excretion of creatinine and the plasma concentration.

3. Comparison of creatinine/inulin clearance ratios determined at different plasma creatinine levels.

1. Comparison of Clearances at Different Plasma Levels.

The relevant data has been summarised in Table 40. The endogenous clearances varied considerably from subject to subject and in order to make the results obtained in different experiments comparable the mean clearances at each level have been expressed as a percentage of the mean endogenous clearance.

At plasma levels of approximately 10 mg./100 ml. the creatinine clearance averaged 103% (78-125%) of the endogenous clearance; at plasma levels of approximately 50 mg./100 ml. the clearances averaged 93% (68-110%) of the endogenous clearance and at plasma levels of approximately 100 mg./100 ml. the clearances averaged 102% (92-106%) of the endogenous clearance. The statistical differences between the mean percentage clearance and 100% has been calculated for levels at which more than four subjects were examined. At no level is the mean percentage clearance significantly different from 100%.

Clearances have also been expressed as a percentage of the mean clearance at a plasma level of about 10 mg./100 ml. The mean value for the endogenous clearance expressed in this way is 99% (80-132%); at a plasma level of approximately 50 mg./100 ml. the mean value is 90% (79-105%) and at a plasma level

of approximately 100 mg./100 ml. it is 94% (84-100%). The difference between the clearances at a level of 10 mg./100 ml. and at a level of 50 mg./100 ml. is statistically significant, (p less than 0.05).

2. The Analysis of the Relationship between UV and P.

The statistical analysis of the relationship between UV and P for creatinine was based on the same considerations as the analysis of the data relating to inulin excretion

Two methods of analysis were employed.

1. Calculation of the linear regression line relating the experimentally determined values of UV and P; calculation of the intercept of this line on the UV-axis and of the error in the estimate of this intercept; determination of the significance of the difference between this intercept and zero.

2. Calculation of the curvilinear regression line $UV = aP^b$; calculation of the error of the estimate of b and determination of the significance of the difference between b and unity.

A summary of the statistics for both these analyses is given in Table 41. From the results of the first method of analysis it is seen that the calculated intercept is not significantly different from zero in any experiment. From the results of the second method of analysis it is seen that the index b is only significantly different from unity in one experiment (subject P). In this experiment b was significantly less than unity, indicating a depression of clearance with increasing plasma level.

The individual results of both regression line analyses with

TABLE 41. A. Creatinine Excretion. Linear Regression Lines fitted to Experimental Data. UV = bP + a.

Subject	Regression Equation	Variance of estimated intercept	t	n -2	P*
W.H. (1)	UV = -0.046 + 1.37 P	0.08	0.15	13	0.9
W.H. (2)	UV = +0.66 + 1.14 P	3.6	0.33	13	0.6
W.H. (3)	UV = +0.50 + 1.11 P	6.2	0.25	6	0.9
W.R.	UV = -0.28 + 1.58 P	1.4	0.23	15	0.8
J.C.	UV = -2.68 + 1.58 P	27.6	0.50	14	0.7
M.	UV = +0.4 + 1.22 P	0.8	0.44	2	0.7
P.	UV = 1.75 + 1.76 P	3.8	0.89	5	0.5
W.	UV = 0.64 + 1.56 P	3.6	0.39	3	0.8

*To be significant p must be less than 0.05.

TABLE 41. B. Curvilinear Regression Lines. $UV = aP^b$

Subject	a	Ccr. when p = 1 mg./100 ml.	b	Variance of b.	t	n-2	P*
W.H. (1)	1.16	116	1.014	0.000163	1.2	13	0.3
W.H. (2)	1.24	124	0.993	0.00104	0.22	13	0.9
W.H. (3)	1.08	108	1.008	0.00086	0.26	6	0.8
W.R.	1.52	152	1.007	0.00026	0.9	15	0.4
J.C.	1.45	145	1.005	0.000096	0.5	14	0.7
M.	1.45	145	0.977	0.00023	1.4	2	0.3
P.	2.14	214	0.923	0.00054	3.5	5	0.02
W.	1.76	176	0.987	0.00057	0.54	3	0.7

* To be significant p must be less than 0.05.

one exception, indicate that the rate of excretion of creatinine is directly proportional to the plasma concentration within the experimental error of the measurements involved.

3. Comparison of Creatinine/Inulin Clearance Ratios at Different Plasma Levels.

The interpretation of the data obtained in these experiments from comparison of clearances at different plasma levels or from regression analysis is largely dependent on the absence of spontaneous changes in renal function during the course of the experiment. In an attempt to remove this difficulty creatinine clearances at different levels were related to simultaneous inulin clearances and the creatinine/inulin clearance ratios compared at different plasma creatinine levels. In doing this, it was assumed that changes in the creatinine clearance unaccompanied by changes in inulin clearance were due entirely to changes in the plasma creatinine concentration and conversely that changes in creatinine clearance accompanied by changes in the inulin clearances were not entirely due to changes in the plasma creatinine concentration. The validity of this argument requires the initial assumption that both inulin and creatinine are excreted mainly as a result of glomerular filtration and that any spontaneous change in overall renal function is indicated by a change in inulin clearance and by a proportionate change in creatinine clearance. With the further assumption that inulin clearance is independent of the plasma concentration, the data from experiments in which only one level of inulin was employed and from those in which two or three levels were employed have been combined.

In view of individual variation in the endogenous creatinine/inulin clearance ratio the ratios have also been expressed as a percentage of the endogenous ratio in order that changes in the ratios may be comparable. In addition the ratios have also been expressed as a percentage of the ratio obtained at a plasma level of about 10 mg./100 ml.

These results are summarised in Table 42 from which it will be seen that the mean ratio has a tendency to decrease as the plasma concentration is increased, being 1.47 at endogenous levels; 1.30 at plasma levels of about 50 mg./100 ml. and 1.22 at plasma levels of about 100 mg./100 ml. The correlation coefficient between the ratio and the plasma concentration for all experiments is -0.61 which is statistically significant. (p less than 0.01). Considering only the experiments in which the plasma inulin concentration was constant the correlation coefficient is -0.62 (p less than 0.02).

There is also a tendency for the ratios expressed as percentage of the endogenous ratio, to decrease as the plasma concentration is increased. The mean percentage ratio at a plasma level of 50 mg./100 ml. is 88% of the mean endogenous ratio but this difference is not significant, (p = 0.1), although apart from one value the percentage ratios are all less than 100%. The mean percentage ratio at plasma levels of 100 mg./100 ml. was 93% of the mean endogenous ratio.

Taking the ratio at plasma levels of 10 mg./100 ml. as the reference ratio the mean ratio at plasma levels of about 50 mg./100 ml. is 10% lower and this difference is statistically significant (p less than 0.02). At plasma level of 100 mg./100 ml.

TABLE 42. Creatinine/Inulin Clearance Ratios (R) and Creatinine Plasma Concentration (Pcr).

Subject	Pcr ≒ 1			Pcr ≒ 10			Pcr ≒ 50			Pcr ≒ 100		
	R	% R Pcr = 1	%R Pcr = 10	R	% R Pcr = 1	% R Pcr = 10	R	% R Pcr = 1	% R Pcr = 10	R	% R Pcr = 1	% R Pcr = 10
*W.H.(1)	1.56	100	98	1.57	102	100	-	-	-	-	-	-
W.H.(2)	1.14	100	82	1.39	122	100	1.26	110	90	1.22	107	88
*W.H.(3)	1.55	100	106	1.46	94	100	1.17	75	80	-	-	-
W.R.	1.56	100	106	1.45	94	100	1.33	86	92	1.19	77	82
J.C.	1.33	100	96	1.39	104	100	1.30	98	94	1.23	92	88
C.M.	1.30	100	-	-	-	-	-	-	-	1.23	94	-
*M.	1.48	100	85	1.74	118	100	1.36	92	78	-	-	-
*P	1.75	100	133	1.31	75	100	1.22	70	93	-	-	-
*W.	1.62	100	116	1.41	86	100	1.44	89	102	-	-	-
Mean	1.47	100	102	1.44	100	100	1.30	88	90	1.22	93	87
‡P	-	-	0.8	-	0.8	-	-	0.1	0.02	-	-	-

* Pin approximately constant throughout.

‡ P = probility of difference from 100 being due to chance alone

the mean percentage ratio is 13% lower.

The ratios as percentage of endogenous ratio and as a percentage of the ratio at plasma levels of about 10 mg./100 ml. have been plotted against plasma concentration (Fig. 11 and Fig. 12). Creatinine/inulin clearance ratios at each level have also been plotted against rate of urine flow (Figs. 13A, 13B and 13C). There is no obvious dependence of clearance ratio on rate of urine flow.

The results of the comparison of creatinine clearance and of creatinine/inulin clearance ratios at different plasma creatinine concentrations suggest that creatinine clearance is dependent to some extent on the plasma creatinine concentration. The results of the regression analysis lead apparently to a conclusion opposed to this and some explanation for this discrepancy is required.

As was discussed previously in considering the excretion of inulin the experimental errors involved in the examination of the excretion of a single substance tend to be relatively large so that the detection of a significant difference between an estimated statistic and a hypothetical value is very difficult. In addition the small total number of collection periods in some experiments also tend to make the variance of the statistics in question large. Thus, although there were with subjects M., P. and W. decreases in the creatinine clearance of 11%, 32% and 8%, respectively, as the plasma creatinine concentration was increased from the endogenous level to levels around 50 mg./100 ml. in all three cases the linear regression line analysis indicated no significant dependence of the clearance on the plasma concentration and, in two out of the three, the curvilinear regression analysis

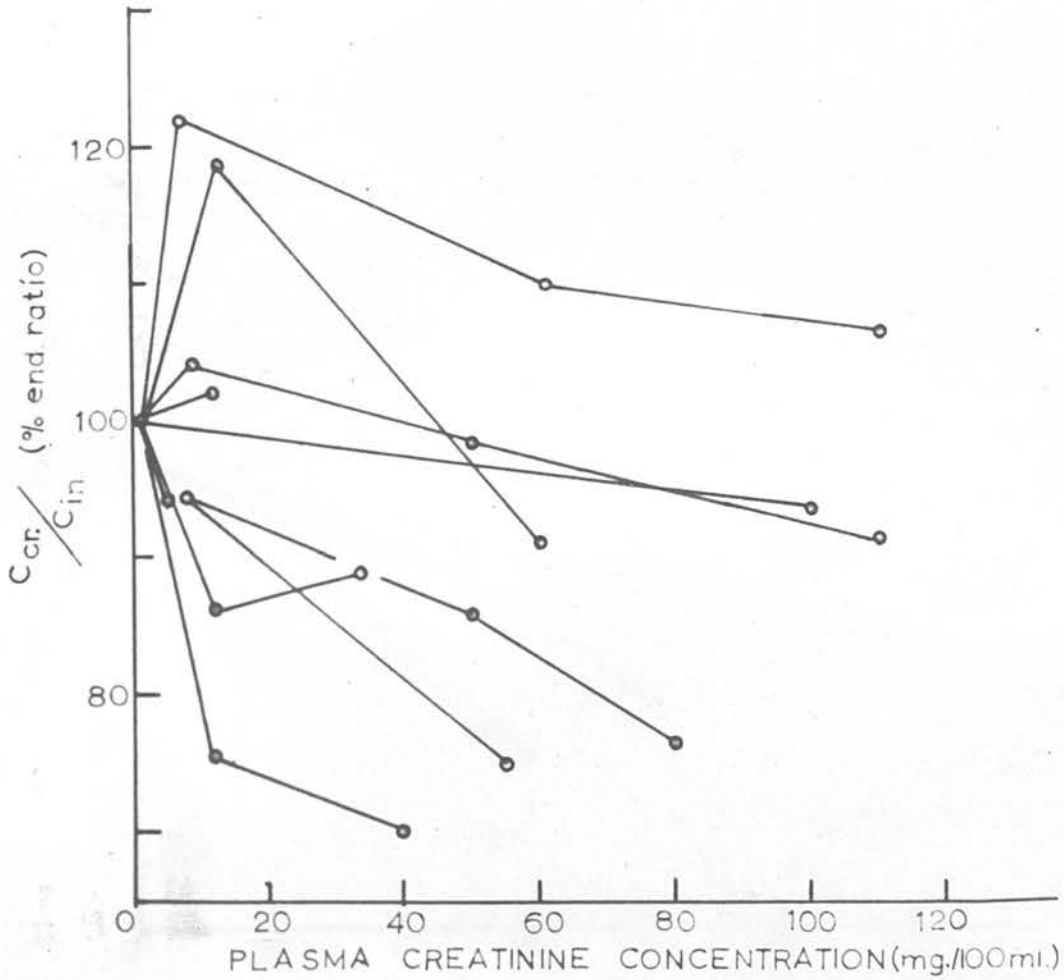

FIGURE 11. Creatinine/inulin clearance ratio and plasma creatinine concentration.

Clearance ratios have been expressed as a percentage of the ratio at endogenous plasma creatinine level.

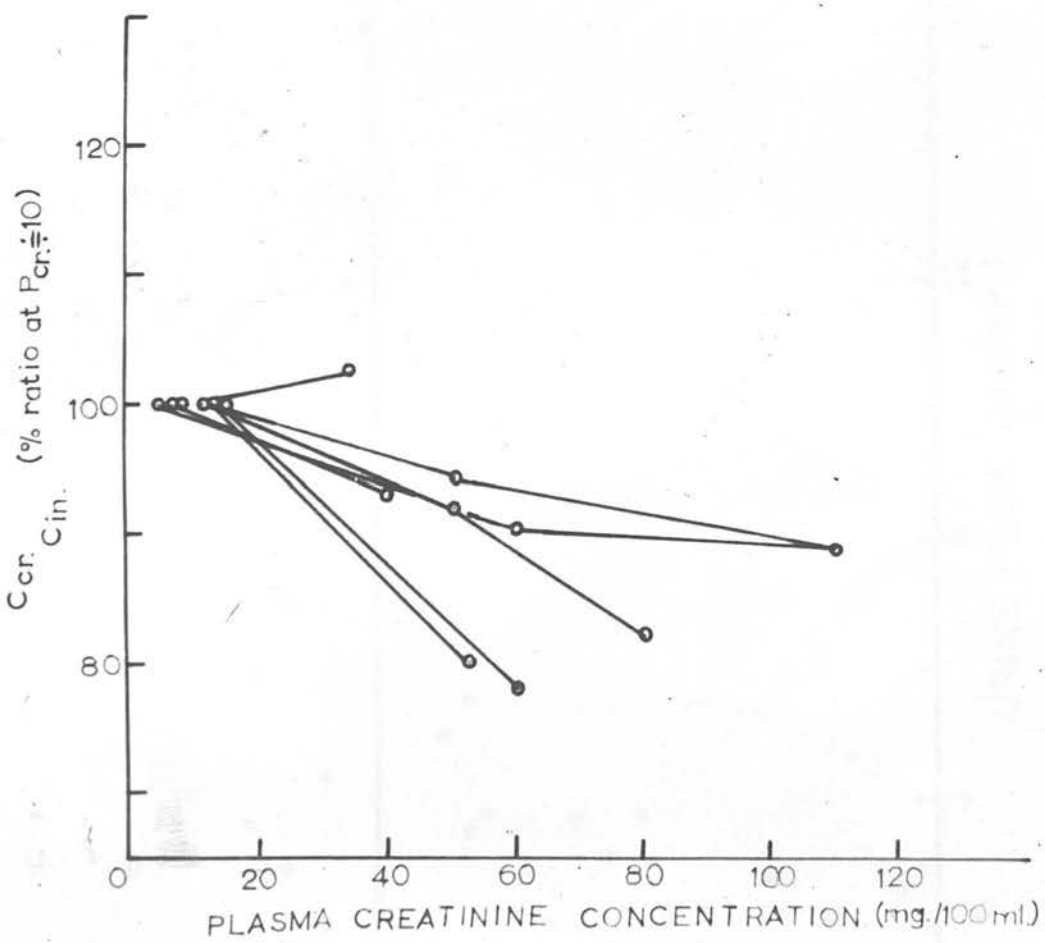

FIGURE 12. Creatinine/inulin clearance ratios and plasma creatinine concentration.

Clearance ratios have been expressed as a percentage of the ratio at plasma creatinine level of about 10 mg./100 ml.

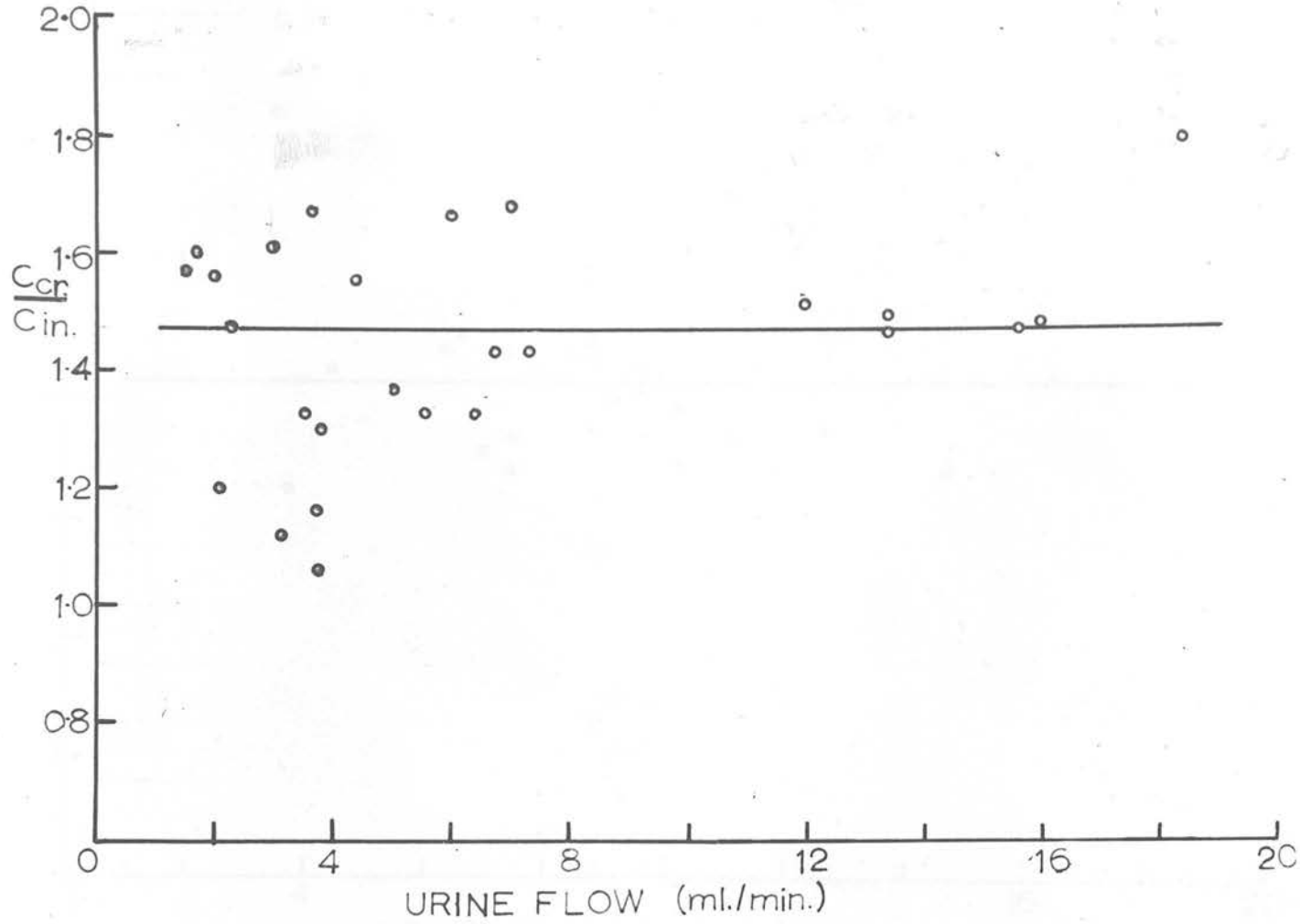

FIGURE 13A. Creatinine/inulin clearance ratios and urine flow. Endogenous plasma creatinine level.

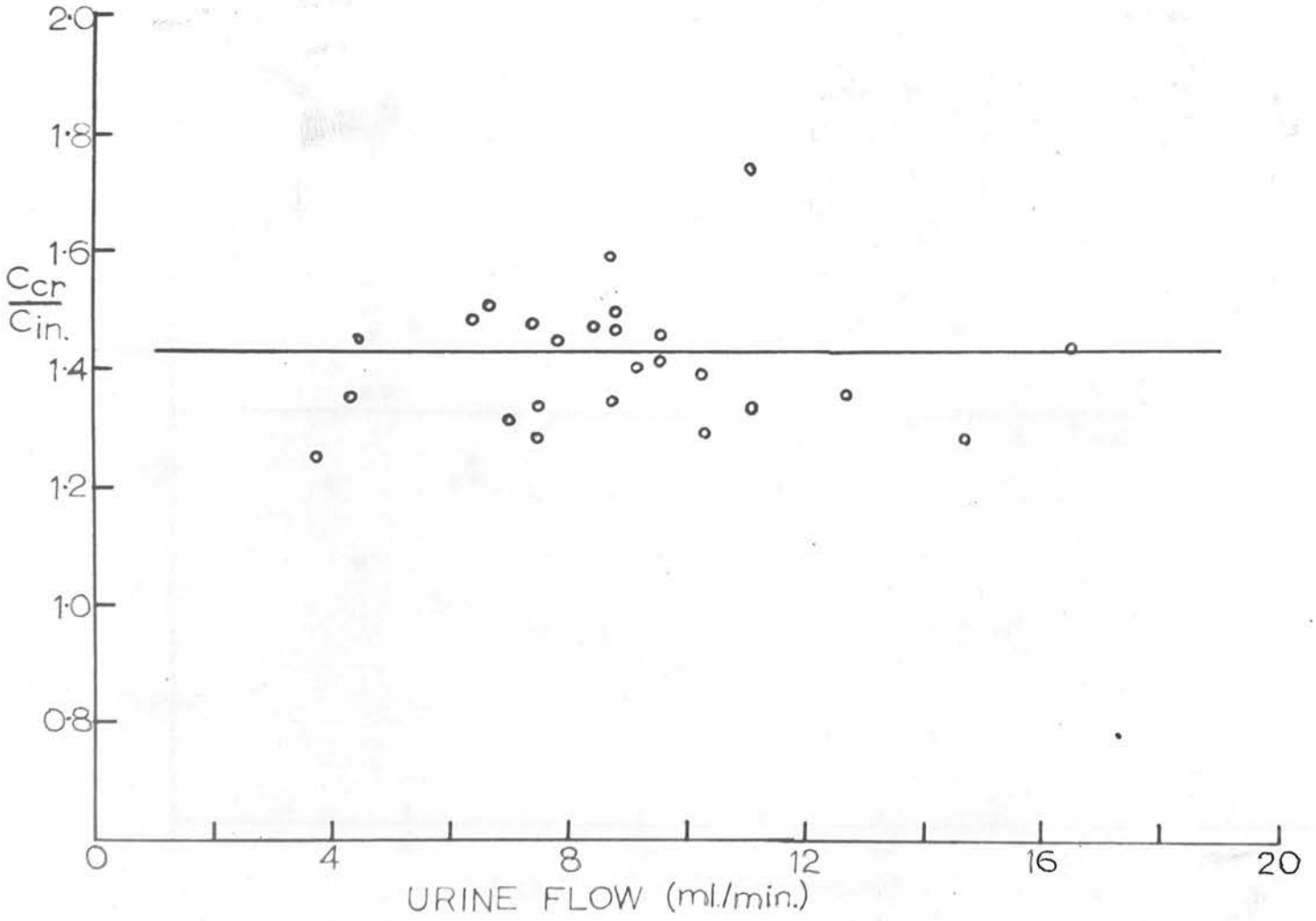

FIGURE 13B. Creatinine/inulin clearance ratios and urine flow. Plasma creatinine levels about 10 mg./100 ml.

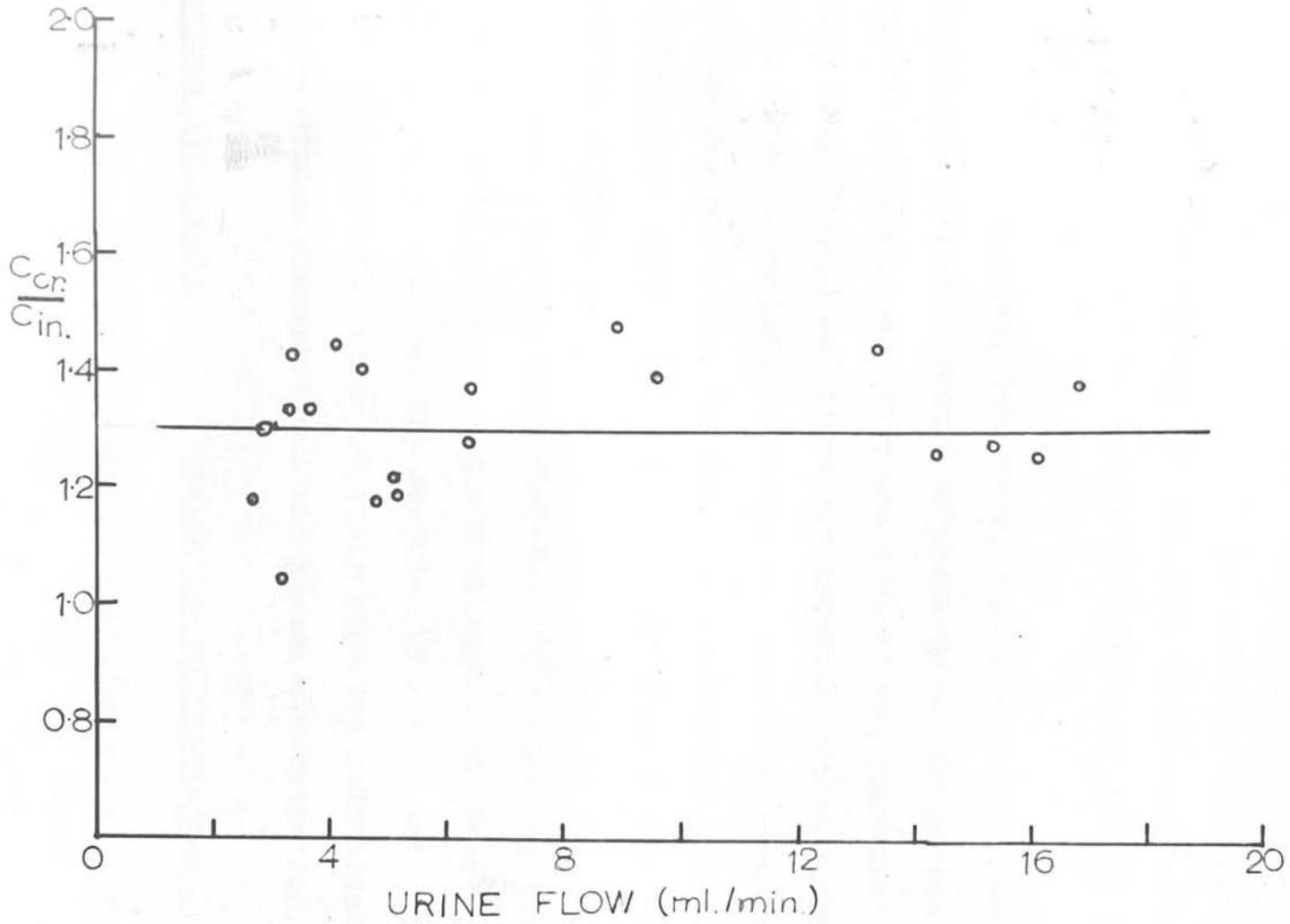

FIGURE 13C. Creatinine/inulin clearance ratios and urine flow.
Plasma creatinine level about 50 mg./100 ml.

indicated likewise.

What is perhaps more important is the fact that with regression line analysis the data relating to each experiment is considered separately whereas with direct comparison of clearances or clearance ratios the data from all the experiments are combined.

It is concluded, therefore, that since the observed creatinine clearance tended to decrease as the plasma concentration was increased (at least when it was increased from 10 to 50 mg./100 ml.) and since the creatinine/inulin clearance ratio showed a definite decrease as the plasma creatinine concentration was increased, the creatinine clearance, as measured by the techniques used, is to some extent dependent on the plasma creatinine concentration.

However, before discussing the implication of this conclusion as such and in relation to the conclusions of others it is appropriate to consider the possible effect of factors which might influence the experimentally observed relationship between the creatinine clearance and the plasma concentration.

Factors influencing the Determination of Creatinine Clearance.

I. The Determination of Creatinine in Plasma, or Serum, and Urine.

The largest error arising out of the method of determination of creatinine will result from the failure to take into account the presence of the non-creatinine chromogen, particularly in plasma or serum. At low plasma levels this results in the overestimation of the plasma creatinine concentration and, since nearly

all the chromogen in urine is creatinine, underestimation of the creatinine clearance. The non-creatinine chromogen is present in plasma or serum in amounts varying from 10-20% of the total endogenous chromogen so that the true endogenous creatinine clearance will be approximately 10-20% higher than the endogenous chromogen clearance.

In the present investigation and in those of Miller and Winkler (1938) and of Hare (1950) specific methods were used for the determination of creatinine at low plasma levels and while the methods which were employed may not be completely specific, the error involved would not appear to be appreciable.

Different methods of preparing protein-free filtrates of plasma or serum are found to give different values for the creatinine content of and for the recovery of creatinine from plasma or serum. (See Section II). Thus "neutral" tungstic acid filtrates (pH greater than 2.5) give values for creatinine content approximately 5% lower than "acid" tungstic acid filtrates (pH less than 2.5). This effect is, however, independent of the plasma concentration so that it should not influence the relationship between the creatinine clearance and the plasma concentration although the absolute value of the creatinine clearance will depend on the type of filtrate used.

2. The Nature of the Chromogen Present in Plasma after the administration of creatinine.

The conversion in the body of administered creatinine to a substance which is chromogenic, and which is excreted by the kidney differently from creatinine, was a hypothesis which was put forward by Shannon and Ranges (1941) to explain the fall in

creatinine clearance which had been noted to occur after the oral administration of creatinine.

Rehberg (1938) had already drawn attention to the claims of Abdon (1937) that after oral administration of creatinine or creatine a labile phorphorus-containing compound resembling phosphocreatine appeared in blood. Shannon and Ranges were, however, unable to obtain any direct evidence for the existence of such a labile phosphorus compound after the oral administration of 10g. creatinine.

To test further this hypothesis creatinine was administered to three normal subjects by mouth and the serum examined for the presence of chromogens other than creatinine.

Experimental.

Serum creatinine concentrations were determined in three normal subjects before and 2 hr. after 5.0 g. creatinine had been taken orally. The NC-bacteria and the Lloyd's reagent methods were used with both "neutral" and "acid" tungstic acid filtrates in order to obtain the true creatinine concentration.

Results.

The results are given in Table 43. It will be seen that all the chromogen present at the higher level, except for a small fraction corresponding to the non-creatinine chromogen normally present, behaved as creatinine.

Discussion and Conclusions.

Chemical evidence of any creatinine-like substance, other than creatinine itself, in plasma after the oral administration

TABLE 43. Chromogens in Plasma 2 hrs. after Oral Creatinine (5g.). All Values expressed as Creatinine.

Subject	Filtrate	Endogenous Level (a) Total Chromogen	(b) NC-bacteria non-creatinine chromogen	(c) (a)-(b)	(d) Lloyd's Reagent	Exogenous Level (e) Total Chromogen	(f)* NC-bacteria non-creatinine chromogen	(g) (e)-(f)	(h) Lloyd's Reagent
		mg./100ml	mg./100ml	mg./100ml	mg./100ml	mg./100ml	mg./100ml.	mg./100ml	mg./100ml
1.	'acid)	0.98	0.31	0.67	0.84	8.00	0.06	7.94	8.00
	'neutral'	0.95	0.23	0.72	0.83	7.60	0.15	7.45	7.62
2.	'acid'	1.07	0.17	0.90	0.92	7.30	0.00	7.30	7.35
	'neutral'	1.06	0.12	0.94	0.85	6.82	0.10	6.72	6.70
3.	'acid'	0.79	0.31	0.48	0.72	7.38	0.0	7.38	7.38
	'neutral'	0.79	0.21	0.58	0.67	6.85	0.0	6.85	7.44

*Owing to the dilutions involved these figures must be regarded as very approximate.

of creatinine was not obtained. Considering this finding in conjunction with that of Shannon and Ranges it would appear that this hypothesis, though considered by Smith (1951) to be likely (he uses it as a criticism of the results of Hare (1950) q.v.) is based almost entirely on physiological evidence of a very indirect and inconclusive nature.

3. The Possibility of Creatinine in Plasma being partly Non-diffusible.

The possibility that creatinine in human plasma is partly non-diffusible due to binding with a non-diffusible substance, e.g. protein, does not seem to have been considered previously to any great extent. Kennedy, Hilton and Berliner (1952) reported that, as shown by in vitro experiments, 5-10% of the creatinine in dog plasma is non-diffusible. The amount bound was found to be proportional to the plasma concentration, in some cases, artificially increased.

The possibility of non-diffusible creatinine existing in human plasma was investigated by a few experiments similar to those carried out by Kennedy et al.

Experimental.

Heparinised plasma, 5.0 ml. and water or creatinine solution 1.0 ml. were placed in dialysis bags made from cellophane tubing. The bags were securely tied off and were placed in test-tubes containing 10.0 ml. isotonic sodium phosphate-sodium chloride buffer solution, pH 7.4. In the tubes containing plasma with no added creatinine, creatinine was added to the buffer

solution, i.e. outside the dialysis bag. Control tubes were also set up in which buffer solution was placed inside the bags instead of plasma. The tubes were then kept for 72 hr. at 0°C. to avoid bacterial decomposition, being shaken intermittently to promote attainment of equilibrium. The bags were then removed from the buffer solutions and an "acid" tungstic acid filtrate was prepared from the plasma. Similar quantities, respectively, of water, sodium tungstate and sulphuric acid were added to an aliquot of buffer from each tube and the creatinine content of the filtrate and of the treated buffer solutions were determined using Lloyd's reagent. In a few tubes traces of protein were found in the buffer and the precipitate which formed on the addition of the above reagents was removed by filtration. In no case was the amount of protein present sufficiently great to indicate significant leakage from the bag. The water content of the plasma was determined by evaporating aliquots to dryness at 100°C.

Results.

These are summarised in Table 44. In every tube except one the concentration of creatinine inside the dialysis bag, calculated in terms of plasma water, was higher than the concentration in the buffer. Three different plasmas gave mean values for the ratio of concentration inside to concentration outside of 1.16, 1.15 and 1.05. There was no consistent tendency for the ratio to depend on the actual concentration nor was there any difference between the ratios in tubes where the creatinine was added to the plasma and those in tubes where

TABLE 44. Diffusibility of Creatinine in Plasma.

Expt.	Creatinine added	Water content of plasma	Creatinine concentration (a) plasma	(b) plasma water	(c) buffer	(b/c)
		%	mg./100ml	mg./100ml	mg./100ml	
1.	None	93.5	0.23	0.25	0.25	1.00
	to plasma	93.2	0.44	0.47	0.32	1.46
		93.5	2.11	2.28	2.00	1.14
		93.2	19.4	21.0	19.8	1.06
	to buffer	93.2	0.63	0.68	0.52	1.71
		93.2	4.24	4.54	3.9	1.14
		92.8	39.5	42.3	43.0	0.99
						Mean 1.16
2.	None	94.7	0.164	0.173	0.156	1.28
	to plasma	94.5	1.04	1.10	0.93	1.18
		94.3	8.80	9.31	8.60	1.08
		94.4	90.8	96.1	85.2	1.13
	to buffer	94.1	1.22	1.29	1.06	1.22
		94.5	9.60	10.2	9.9	1.03
		94.5	113.2	121	76.8	1.58
						Mean 1.15
3.	None	94.3	0.188	0.201	0.188	1.07
	to plasma	94.2	1.07	1.15	1.07	1.07
		94.0	8.64	9.33	8.72	1.07
	to buffer	94.1	1.20	1.28	1.20	1.07
		94.1	9.52	10.3	9.80	1.05
						Mean 1.07

the creatinine was added to the buffer. In the control tubes the concentrations of creatinine in the buffer inside and outside the bag were always identical.

Discussion and Conclusion.

These results suggest that, in vitro, a fraction of the creatinine in plasma cannot diffuse through a membrane which is impermeable (or very slightly permeable) to protein but which is freely permeable to creatinine in the absence of plasma. If this is applicable to plasma in vivo, and if the permeability of the cellophan membrane is similar to that of the glomeruli, the concentration of diffusible creatinine in plasma must, in fact, be less than the total creatinine concentration so that the creatinine clearance calculated from the latter underestimates the true creatinine clearance to the extent of 10-15%. However, since the apparent binding observed was independent of the actual concentration the relationship between clearance and plasma concentration remains unaffected.

General Discussion of the Relationship between the Creatinine Clearance and the Plasma Concentration.

The present findings suggest that, in the majority of cases, the creatinine clearance decreases as the plasma creatinine concentration is increased from endogenous plasma creatinine levels of 50-100 mg./100 ml. This finding is in agreement with that of Shannon (1935), although quantitatively the depression of the creatinine clearance observed in the present series of experiments was less than that observed by him, but is contrary to

to that of Miller and Winkler (1938). It is necessary, therefore, to consider possible explanations for the phenomenon and reasons for the discrepancies.

Errors in the chemical determination of the diffusible creatinine in plasma may arise from several sources, e.g. in preparation of protein-free filtrates of plasma, or from failure to control adequately the colour reaction. Although such errors undoubtedly exist and may cause errors in the absolute value of the creatinine clearance, the present evidence suggests that these errors are independent of the plasma concentration and, therefore, do not affect the relationship between the creatinine clearance and the plasma concentration. In addition there is no satisfactory evidence to suggest that after the administration of creatinine the non-creatinine-chromogen content in the plasma increases.

At plasma levels below 10 mg./100 ml. errors in the determination of the true plasma creatinine concentration may arise as a result of the poor specificity of the methods used. There is no evidence to suggest, however, that the use of the NC-bacteria or of Lloyd's reagent, introduces an appreciable error. Nevertheless, it is possible that the larger variation in the creatinine clearance at endogenous levels compared with that in clearances at exogenous levels is due to greater errors in the determination of creatinine in serum and urine at endogenous levels.

Apart from the explanation originally put forward by Shannon, i.e. depression of clearance at high plasma levels due to saturation of tubular excretion, physiological explanations

for the phenomenon of self-depression include delay-time, the possibility of diffusion of creatinine from the tubules and the possibility of creatinine being formed by the kidney and excreted in the urine.

In considering the effect of delay-time on inulin clearance it was noted that delay-time clearance anomalies are present after a single injection of a substance even when this is followed by a continuous infusion. Furthermore, in the two experiments carried out by the present author to investigate delay-time effects the data suggested that delay-time anomalies are greater with creatinine than with inulin, possibly as a result of the more rapid decrease in plasma concentration.

Shannon's values for the creatinine/inulin clearance ratios were obtained while the plasma concentration was decreasing and, for this reason, may have been erroneously high. In the present investigation the administration of a continuous infusion after the priming injection could explain the finding of relatively less depression of the creatinine/inulin clearance ratio at high plasma levels than was found by Shannon, since the effect of delay-time is presumably less when an infusion immediately follows the injection. It would seem, however, very unlikely that a cumulative delay-time effect could produce a progressive fall in clearance during several hours and thus be responsible for the entire phenomenon of self-depression.

On the other hand, it is likely that delay-time effects were partly responsible for the finding by Miller and Winkler (1938) of an exogenous (Pcr = 20 mg./100 ml.)/endogenous creatinine

clearance ratio of 1.4, since these authors started to measure exogenous creatinine clearances shortly (20 min.) after the primary injection. This is further suggested by the fact that in the one experiment in which there were several exogenous collection periods the creatinine/inulin clearance ratio showed a progressive decline although unity was never reached. In most of these experiments the exogenous clearances were only measured for a short time and the maximum possible decreases in the creatinine/inulin clearance ratio may not have been reached.

It would seem also possible that the endogenous creatinine clearances of Miller and Winkler were erroneously low since the mean endogenous creatinine/inulin clearance ratio found, i.e. 1.13, was lower than that subsequently reported by the same group, i.e. 1.25 (Taggart et al., 1952). Both of these explanations would reduce the discrepancy between the present findings and those of Miller and Winkler.

Back diffusion of creatinine from the tubules to the plasma taking place, to a greater extent when the concentration in the tubular fluid is high, has been suggested as an explanation of the self-depression of creatinine clearances (Findley, 1937; Ekehorn, 1944). The extent to which back diffusion takes place would presumably also depend on the rate of urine flow.

Passive back diffusion of urea taking place to a greater extent at low rates of urine flow has been postulated as an explanation for the direct dependence of urea clearance on the

rate of urine flow. This dependence is most marked when the latter is below 2 ml./min., but occurs to a smaller extent at higher rates of urine flow. A qualitatively similar dependence of inulin clearance and exogenous creatinine clearance on the rate of urine flow has been reported (Holden and Bulger, 1946) but this has generally been attributed to changes in the actual glomerular filtration rate associated with the changes in the rate of urine flow (Smith, 1951). Shannon (1936) reported that in the dog the exogenous creatinine (Pcr = 10 mg./100 ml)./inulin clearance ratio was independent of the urine flow as low as at corresponding to a urine-plasma concentration ratio of 500 and concluded in view of the different diffusion coefficients of these two substances that reabsorption of either from the tubules by passive diffusion did not take place.

In the present series of experiments there was no obvious dependence of the creatinine/inulin clearance ratio on the rate of urine flow at any plasma level (Fig. 13A,B & C). It must be noted, however, that rates of urine flow below 2 ml./min. were seldom observed so that the possibility of back diffusion of creatinine cannot be entirely excluded.

The formation in the kidney and direct excretion of a significant fraction of the urinary creatinine at endogenous plasma levels would account for a depression of the creatinine clearance as the plasma creatinine concentration. Such a process could be detected by comparison of the rate of urinary excretion of creatinine with the renal arterio-venous extraction

rate. However the absence of consistent depression of the creatinine clearance until the plasma level is increased to above 10 mg./100 ml. renders this explanation unlikely.

In summary, therefore, it is concluded that when the plasma creatinine concentration is artificially increased the creatinine clearance decreases; that this depression is only appreciable at plasma levels higher than 10 mg./100 ml.; and that the most likely explanation for this finding is the saturation of a tubular mechanism for the excretion of creatinine.

The Relationship between the Renal Clearances of Creatinine and Inulin.

If the mechanism by which a substance is excreted by the kidney is known, comparison of the simultaneous clearance of this substance with that of a second substance may give information about the renal excretion of the second substance. If, for example, one substance is believed to be excreted solely by glomerular filtration, then a substance which has a clearance less than the reference substance may be assumed to be reabsorbed by the renal tubules; and one which has a clearance more than the reference substance is assumed to be excreted by the renal tubules. In practice, however, this principle is not of great use since the mechanism by which the kidney handles the reference substance is never known for certain. In all such comparisons it is essential that the two substances

do not interfere with their respective excretions, a requirement which many pairs of substances do not satisfy.

Following the claims of Shannon and Smith (1935) that inulin was excreted solely by a process of filtration the ratio of the simultaneously determined creatinine and inulin clearances has been examined in attempts to gain information about the renal excretion of creatinine. Comparisons have been made under a variety of conditions and both endogenous and exogenous creatinine clearances have been compared with inulin clearances.

In the interpretation of clearance ratios factors which influence the determination of the inulin clearance must be considered in addition to the factors influencing the determination of the creatinine clearance. The method of the administration of inulin and the method of determination are the most important additional factors.

For the purpose of description it has been found convenient to classify the data from the literature as follows.

1. Clearance ratios in adults with normal renal function obtained at endogenous plasma creatinine concentrations, using non-specific methods for the determination of creatinine.
2. Clearance ratios in adults with normal renal function obtained at endogenous plasma creatinine concentrations using specific methods for the determination of creatinine.
3. Clearance ratios in adults with normal renal function obtained at exogenous plasma creatinine concentrations.
4. Clearance ratios in children.
5. Clearance ratios in subjects with renal disease.

1. Clearance ratios in adults with normal renal function obtained at endogenous creatinine concentrations using non-specific methods for the determination of creatinine.

Shannon (1936) in his studies on the excretion of creatinine at different plasma concentrations did not report the endogenous chromogen clearance for the four subjects studied although he gave the endogenous chromogen clearances of four other subjects. However by extrapolation of data he concluded that the true endogenous creatinine/inulin clearance ratio would be about 1.4.

Smith, Finkelstein and Smith (1940), using picric acid filtrates for endogenous chromogen determination in serum obtained a mean ratio of 1.12 (1.01 - 1.22) in three normal subjects. Using ferric carbonate filtrates a mean ratio of 0.97 (0.82 - 1.02) was obtained in four normal subjects. Steinitz and Turkand (1940) using picric acid filtrates reported a mean ratio of 1.03 (0.73 - 1.17). Brod and Sirota (1948) using "neutral" tungstic acid filtrates reported a mean ratio of 1.00 (0.88 - 1.10) in 14 normals. Blegen, Haugen and Aas (1949) using "neutral" tungstic acid filtrates reported a mean ratio of 0.84 (S.D. = 14). Brod and Kotatko (1949) using "acid" tungstic acid filtrates reported a mean ratio of 0.66 (0.52 - 0.91), determined while PAH was present in the plasma in a concentration of 1-2 mg./100 ml. Mandel et al. (1953) using both "neutral" and "acid" tungstic acid filtrates found a mean ratio of 0.76 (S.D.O. 065). Roscoe (1953) using "acid" tungstic acid filtrates found a mean ratio

of (0.71 - 1.08). (The actual figures were not reported but these ratios have been calculated, approximately from a graph relating endogenous chromogen and inulin clearances). Doering, Schroeder, Schubert and Schwab (1953) obtained a mean ratio of 1.02 using picric acid filtrates and a mean ratio of 0.79 using "acid" tungstic acid filtrates.

Sirota, Baldwin and Villareal (1950) measured clearances over 24 hr. in four-hourly periods. They found a mean ratio of 0.95 (0.71 - 1.09). Sims and Seldon (1949) reported an endogenous chromogen/mannitol clearance between 0.82 and 1.34. The mannitol/inulin clearance ratio is at present believed to be about 0.9 (Barger, Farber and Earle, 1947; Corcoran and Page, 1947; Smith, 1951), so that the values found by Sims and Seldon expressed as endogenous chromogen ratios become approximately 0.7 - 1.2.

These results have been summarised in Table 45.

2. Clearance ratios in adults with normal renal function at endogenous creatinine concentrations using specific methods for creatinine determination.

Miller and Winkler (1940), using the NC-bacteria with "neutral" tungstic acid filtrates for the determination of creatinine obtained a mean endogenous creatinine/inulin clearance ratio of 1.13 (0.8 - 1.5). Later Tagart, Mamby and Miller (1952) reported that the ratio was always higher than 1.0 in normals. In a series of normals and hypertensives they found a mean ratio of 1.25.

Barclay and Kenney (1947), using a nephelometric method for the determination of creatinine in serum and urine obtained a mean creatinine clearance approximately 30% higher than the mean inulin clearance.

Brod and Kotatko (1949), using Lloyd's reagent with "acid" tungstate filtrates obtained a mean ratio of 1.03 (0.82 - 1.58) in a series of patients with no evidence of renal disease.

Hare (1950) using Lloyd's reagent with trichloracetic acid filtrates obtained a mean ratio of 1.03 (0.82 - 1.26) in a series of 22 normals including children.

Haugen and Blegen (1953) using Lloyd's reagent applied to "neutral" tungstic acid filtrates obtained a mean ratio of 1.01 (0.87 - 1.17) in a series of 18 subjects, of which 10 were normal and the rest had mild or moderate chronic nephritis.

Mandel et al. (1953) using Lloyd's reagent with "acid" tungstic acid filtrates obtained a mean ratio of 1.04.

These results have been summarised in Table 46. It is rather surprising that the range of mean ratios reported by workers using specific methods for creatinine determination is very similar to the range of mean ratios obtained with non-specific methods, in view of the fact that the latter ratios are known to be 10-20% too low.

3. Clearance ratios in adults with normal renal function, at exogenous creatinine concentrations.

Shannon (1935) reported that as the plasma concentration of creatinine was raised the ratio decreased from a mean value

of 1.4 at a plasma level of 10 mg./100 ml. (P_{cr} = 10) to a mean value of 1.2 (P_{cr} = 100). As the plasma concentration decreased from the highest level the ratio remained constant. (This finding has already been discussed). McCance and Widdowson (1937) using single injections of creatinine (P_{cr} = 10) and inulin obtained ratios of 1.29 and 1.5 in two normal subjects. Miller and Winkler (1938) using continuous infusion of creatinine and inulin obtained a mean ratio of 1.6 (1.1 - 2.0) (The possibility that these results are too high has also been discussed previously). Shannon and Ranges (1941) obtained a mean ratio of 1.4 using continuous infusions of creatinine and inulin (P_{cr} = 20). After oral creatinine the mean ratio, initially 1.45 (P_{cr} = 12), decreased with decreasing plasma concentrations to a mean value of 1.15 (P_{cr} = 6).

Josephson and Lindahl (1943) using single injections of inulin and oral creatinine obtained ratios greater than 1.0. Crawford (1948) using continuous infusions of creatinine and inulin (P_{cr} = 8-12) obtained a mean ratio of 1.25 (1.1 - 1.3). Hogeman (1943) using single injections of inulin and oral creatinine (P_{cr} not stated) obtained a mean creatinine clearance of 15%. Bucht (1949) using oral creatinine (P_{cr} = 5-8) and infusions of inulin obtained a mean ratio of 1.24 (S.D. 0.07). Hare (1950) obtained a mean rate of 1.08 (0.92 - 1.20) (P_{cr} and method of administration not stated). Mandel et al. (1953) using infusions of inulin obtained a ratio of 1.74 and 1.15 in two subjects after oral creatinine (P_{cr} = mg./100 ml.)

These results have been summarised in Table 47.

4. Clearance ratios in infants and children with normal renal function.

Brod and Sirota (1948) obtained a mean endogenous chromogen/mannitol clearance ratio of 0.60 (0.55 - 0.69) in four infants aged 2-25 months. (Figures for the mannitol/inulin clearance ratio in infants are not available; in adults the ratio is about 0.9). Doxiadis and Goldfinch (1952) obtained a mean endogenous chromogen/inulin clearance of 0.54 (0.34 - 0.60) in children under two years old and a mean ratio of 0.56 (0.49-0.66) in children aged from 2-11 yrs.

Barnett, Hare and McNamarra (1949) using an unstated method (probably Lloyd's reagent) for creatinine determination reported that the ratio was less than 1.0 in infants but increased with age until, when inulin clearances reached values above 80 ml./min. the ratio was greater than 1.0. Calcagno and Rubin (1951) using Lloyd's reagent applied to an "acid" tungstic filtrate obtained a mean ratio of 0.8 (0.55 - 1.22).

Lauson (1951) reported that the non-creatinine chromogen content of plasma in children determined with NC-bacteria was relatively greater than that of plasma in adults. His figures obtained with Lloyd's reagent show that the total non-creatinine chromogen in plasma is about the same in children as in adults, i.e. about 0.2 mg./100 ml. (as creatinine). Correcting the figures of Brod and Sirota and of Barnett et al., ($P_{chr.}$ = 0.4 - 0.6) still gives mean ratios below 1.0.

Emmerson, Futchev and Farr (1940) using oral creatinine

(P_{cr} = 10) found ratios of 1.50 and 1.32 respectively in two children. Dean and McCance (1947), using infusions of creatinine (P_{cr} = 10) and inulin, found a mean ratio of 0.98 (0.76 - 1.32).

These results have been summarised in Table 48.

5. Clearance ratios in subjects with renal disease.

Numerous investigators have measured creatinine/inulin clearance ratios in subjects with renal pathology and the results of these ingestigations tend to be just as variable as those reported for the ratio in subjects without normal renal function. No useful purpose is served by discussing in detail these results which have been summarised in Tables 49-51, in which they have been grouped in the same way as those relating to subjects with normal renal function. In the first group, in which creatinine clearance was measured at endogenous levels using non-specific methods the mean ratios range from 0.76 - 1.38. In considering these figures it should be noted that the findings of Brod and Kotatko (1949), and Miller et. al. (1952) suggest that in renal disease the non-creatinine chromogen content is increased disproportionately less than the true creatinine content.

In the second group where specific methods were used for the determination of creatinine at endogenous levels the mean ratios vary from 1.38 - 1.50 (range of individual ratios 0.9 - 2.2).

In the third group in which exogenous creatinine clearances

TABLE 45A.

Abbreviations used in Tables 45-53.

NS - not stated.

Administration of Creatinine & Inulin.

Inf. - continuous infusion.

S.I. - single injection.

Method of Creatinine Determination.

PA - picric acid filtrate.

FC - ferric aarbonate filtrate.

NT - "Neutral" tungstate filtrate.

AT - "Acid" tungstate filtrate.

Cd - Cadmium hydroxide filtrate.

TCA - Trichloracetic acid filtrate.

NC-B - NC-bacteria.

LR - Lloyd's reagent.

TABLE 45. Comparison of Creatinine and Inulin Clearances in Subjects with Normal Renal Function. Endogenous Levels. Non-Specific Method. Summary of Literature.

Author	No. of Subjects	Pcr	Reference Substance	Method of Creatinine Determination	Ccr/Cin Mean & Range
Smith et al.,1940	3	Endogenous	Inulin Inf.	PA	1.12(1.0-1.6)
	4	"	" "	FC	0.98(0.8-1.1)
Steinitz & Turkand,1948	-	"	" "	PA	1.03(0.73-1.17)
Brod & Sirota,1948	14	"	" "	AT	1.00(0.88-1.10)
Blegen et al.,1948	15	"	" "	NT	0.84(S.D. = 0.14)
Brod & Kotatko,1949	11	"	" "	AT	0.66(0.52-0.91)
Sims & Seldon,1949	-	"	Mannitol Inf.	NT	0.82-1.34 ($\equiv$ 0.7-1.2 as $c\frac{C_{chr}}{In}$)
Sirota et al,1950	6	"4hrly	Inulin Inf.	NT	0.93(0.71-1.09)
Mandel et al,1953	5	"	" "	NT & AT	(0.65-0.86) 0.76(S.D. = 0.065)
Roscoe,1953	13*	"	"	AT	0.84(0.71-1.08) from graph.
Doering et al,1953	10	"	" Inf.	PA	1.02(SE.= 0.013)
	17	"	" "	AT	0.79(SE.= 0.026)

* including 4 subjects with renal failure.

TABLE 46. Comparison of Creatinine and Inulin Clearances in Adults with Normal Renal Function Using Specific Methods for Determination of Creatinine at Endogenous Levels.

Author	No. of Subjects	Creatinine Plasma Level	Reference Substance	Method of Creatinine Determination	Ccr/Cin. Mean & Range
Shannon, 1935	4	Endogenous	Inulin SI	FC	1.4 (extrapolation)
Miller & Winkler, 1938	7	"	" Inf.	NT NC-B	1.13 (0.8-1.5)
Barclay & Kenney, 1947	N.S.	"	" N.S.	Nephelometric	$\frac{\text{Mean Ccr}}{\text{Mean Cin}} \doteqdot 1.3$
Brod & Kotatko, 1945	24	"	" Inf.	AT LR	1.03 (0.82-1.58)
Hare, 1950	22 *	"	" N.S.	TCA LR	1.03 (0.82-1.26)
Taggart et al., (unpub)	N.S.	"	" N.S.	NT NC-B	1.25 (all 1.0)
Haugen & Blegen, 1953	18 †	"	" Inf.	NT LR	1.01 (0.87-1.17)
Mandel et al., 1953	13	"	" Inf.	AT LR	1.04 (S.D. = 0.12)

For abbreviations see TABLE 45a

* including children.

† including some subjects with renal disease.

TABLE 47. Comparison of Creatinine and Inulin Clearance in Adults with Normal Renal Function at Exogenous Plasma Creatinine Levels.

Author	No. of Subjects	Creatinine Plasma Level mg/100 ml.	Reference Substance	Method of Creatinine Determination	Ccr/Cin Mean & Range
Shannon, 1935	4	Oral 10-20 SI 50-100	Inulin SI	FC	1.4(1.3-1.45) 1.3 1.15
McCance & Widdowson, 1937	2	SI 10	Inulin SI	?	1.3 & 1.5
Winkler & Para, 1937	14	Oral & SI 4-25	Sucrose SI	NT	(1.0-2.5)
Miller & Winkler, 1938	4	Inf. 10-20	Inulin Inf.	NT	1.6 (1.1-2.0)
Shannon & Ranges, 1941	6	Oral 6-12) Inf. 10)	Inulin Inf.	NT	(1.45 1.15 (1.4
Hogeman, 1943	21	Oral N.S.	Inulin SI	NT	Mean Ccr / Mean Cin = 1.16
Crawford, 1948	7	Inf. 8-12	Inulin Inf.	Cd.	1.25(1.1-1.3)
Bucht, 1949	8	Oral 5-8	Inulin SI & Inf.	NT	1.24 (S.D. = 0.07)
Hare, 1950	22	NS. NS.	Inulin NS	TCA LR	1.08 (0.97-1.20)
Mandel et al., 1953	2	Oral 3 & 6	Inulin Inf.	TCA AT LR	1.74 & 1.15

TABLE 48. Comparison of Creatinine and Inulin Clearances in Infants and Children with Normal Renal Function.

Author	No. of sub-jects	Age	Creatinine Plasma Level	Inulin	Method of Creatinine Determination	Ccr/Cin.
Brod & Sirota, 1948	4	2-25 mths	Endog.	(Mannitol) Inf.	AT.	0.60(0.55-0.69)
Barnett et al., 1949	-	various	Endog.	Inulin	N.S.	< 1.0 infants becoming > 1.0 with age
Calcagno & Rubin, 1951	4	-	Endog.	Inulin	TCA. LR	0.8(0.55-1.22)
Doxialis & Goldfinch, 1953	11 9	< 2 yrs > 2 yrs	Endog.	Inulin	AT	0.54(0.34-0.60) 0.56(0.49-0.66)
Emerson et al., 1940	2		Oral $P_{cr.}$= 10	Inulin S.I.		1.32 & 1.50
Dean & McCance, 1947	6	1 wk.	Inf. $P_{cr.}$= 10	Inulin Inf.		0.98(0.76-1.32)

TABLE 49. Comparison of Creatinine and Inulin Clearances in Persons with Various Diseases. Non-specific Methods at Endogenous Levels.

Author	No. of Subjects	Diagnosis	Creatinine Plasma Level	Reference Substance & Administration	Method of Creatinine Determination	Ccr./Cin. Mean Range
Emmerson et al. (1940)	2	Nephrosis	Endog. Pcr. = 2 & 4	Inulin SI	NT	1.38 &1.37
Smith etal. (1940)	2	Essential Hypertension	Endog.	Inulin Inf.	PA FC	1.34(1.20-1.42) 0.94
Steinitz & Turkand (1940)	24	Various renal diseases	Endog.	Inulin Inf.(?)	PA	1.37(1.04-1.73)
Baldwin et al. (1948)	8	Congestive heart failure	Endog.	Inulin Inf.	NT	0.90(0.78-1.06) 0.76(0.65-0.81)
Blegen et al. (1949)	27	Cardiac failure	Endog.	Inulin Inf.	NT	0.89(SD 0.16)
Brod & Kotatko (1949)		Various	Endog.	Inulin Inf.	AT	(0.6-1.6)
Baldwin (unpub.: Smith, 1951)	NS	Various	Endog.	N.S.	AT	0.84(0.65-1.03)
Haugen, (1951)	4	Ureamia	Endog.	Thiosulphate Inf.	NT	1.36(SD 0.20)
Miller et al. (1952)	9	Various	Endog.	Inulin Inf.	NT	(0.56-1.6)
Doxialis & Goldfinch (1952)	3	Various - children	Endog.	Inulin SC	AT	(0.88-0.78)
Mandel et al. (1953)	10	Various	Endog.	Inulin Inf.	NT & AT	1.29(0.90-1.86)

TABLE 50. Comparison of Creatinine and Inulin Clearances in Persons with Various Diseases. Specific Methods at Endogenous Levels.

Author	No. of Subjects.	Diagnosis	Creatinine Plasma Level	Reference Substance & Administration	Method of Creatinine Determination	Ccr/Cin. Mean	Ccr/Cin. Range
Miller & Winkler, 1938	4	Renal disease	Endogenous	Inulin Inf.	NT NC-B	1.38	(0.9-1.7)
Brod & Kotałko, 1949	9	Various	"	" "	NT LR	1.50	(1.07-2.2)
Hare et al., 1949	NS	"	"	Inulin ? Inf.	TCA LR	1.29	- 2.64
Mattar et al., 1952	3	Nephritis (children)	"	Inulin Inf.	TCA LR	1.46	(1.18-2.01)
Miller et al., 1952	9	Renal disease	"	" "	NT NC-B	1.31	(0.83-1.71)
Mandel et al., 1953	21	Various	"	" "	TCA & AT) LR	1.25	(0.72-1.83)

TABLE 51. Comparison of Creatinine and Inulin Clearances in Persons with Various Diseases. Exogenous Creatinine Levels.

Author	No. of Subjects	Diagnosis	Creatinine Administered	Reference Substance	Method of Creatinine Determination.	Ccr/Cin
Winkler & Para, 1937	22	glomerulo-nephritis	Oral S.I.	Sucrose S.I.		(1.1-3.3)
Miller & Winkler, 1938	4	renal disease	Infusion	Inulin Inf.	NT	(1.0-1.9)
Emmerson et al., 1940	8	nephrosis	Oral	Inulin S.I.	NT	1.35(1.13-1.61)
Steinitz & Turkand, 1940	12	various	Oral	Inulin Inf.	PA	1.25
Josephson & Godin, 1943	N.S.	various	Oral	Inulin S.I.	NT	Ccr = Cin + 30
Hogeman, 1943	-	various	Oral	Inulin S.I.	NT	> 1.0
Hare et al., 1949	N.S.	various renal diseases	Oral and Intravenous	Inulin N.S.	TCA LR	(1.29-2.64)

were measured the mean ratios are not available for all investigations. Ratios in individual subjects vary from 1.0 - 2.6.

Summary of comparisons of creatinine and inulin clearances from literature.

The variability of the findings reported in the literature make a concise summary of all the data impossible. There are however three main conclusions which may be drawn.

1. The mean endogenous chromogen/inulin clearance ratio reported for normal adults tends to be equal to or rather less than 1.0; the mean endogenous creatinine/inulin clearance ratio reported tends to be equal to or greater than 1.0; and the mean exogenous creatinine/inulin clearance ratio reported is always greater than 1.0.

2. In children the endogenous chromogen/inulin clearance and the endogenous creatinine/inulin clearance ratio are both less than 1.0.

3. In subjects with renal disease the creatinine/inulin clearance ratio tends to be considerably greater than 1.0.

In the course of this investigation simultaneous inulin and creatinine clearances were determined in a number of subjects including normals and subjects with various diseases, generally associated with functional or pathological renal dysfunction. Inulin clearances were determined by the single injection technique devised by Robson et al. (1949) or, in a few subjects, by the continuous infusion method. Creatinine

in serum and urine was determined using both specific and non-specific methods, and both endogenous chromogen and true endogenous creatinine clearances were determined.

The results for normal subjects are given in Table 52. The mean chromogen/inulin clearance ratio was 1.18 and the mean creatinine/inulin clearance ratio was 1.44. No distinction has been made between the results obtained in subjects given a single injection of inulin and in those given an infusion of inulin since the mean ratios obtained by grouping the results accordingly are not significantly different.

The creatinine/inulin clearance ratios at exogenous creatinine levels have already been discussed and are summarised in Table 42. At all levels the mean ratio was greater than unity, being 1.44 at levels of 10 mg./100 ml., 1.30 at levels of 50 mg./100 ml. and 1.22 at a level of 100 mg./100 ml.

The endogenous chromogen, endogenous creatinine and inulin clearances in subjects with various diseases are summarised in Table 53. The mean chromogen/inulin clearance ratio was 1.23 and the mean creatinine/inulin clearance ratio was 1.62. The diagnoses in the "abnormal" series were however very varied and the limited numbers exclude the possibility of a detailed analysis of the results in relation to renal pathology.

Regression analysis of the relationship between creatinine and inulin clearances.

The expression of the relationship between creatinine and inulin clearances as a mean ratio does not show any dependence

TABLE 52. Endogenous Creatinine/Inulin Clearance Ratios in Normal Persons.

Clearances not corrected to a standard surface area.

Age	Sex	Inulin		Creatinine		Ccr/Cin.	
		Administration	Clearance	Clearance (Uncorr.)*	Clearance (Corr.)	Uncorr.	Corr.
			ml./min	ml./min	ml./min		
26	M	S.I.	111	120	144	1.07	1.31
51	M	S.I.	133	139	169	1.04	1.26
52	M	S.I.	80	107	131	1.34	1.63
56	M	S.I.	73	88	115	1.21	1.57
25	M	S.I.	108) 109	121) 130	155) 158	1.12)	1.31)
25	M	S.I.	110)	139)	161)	1.27)	1.46)
19	M	Infusion	114	159	200	1.39	1.75
20	M	Infusion	93	112	136	1.21	1.47
24	M	Infusion	114	128	175	1.12	1.53
32	M	Infusion	87)	107)	134)	1.22)	1.55)
"	"	Infusion	99) 93	90) 104	112) 127	0.91)	1.11)
"	"	Infusion	75)	100)	116)	1.33)	1.55)
"	"	Infusion	109)	120)	148)	1.10)	1.36)
Mean			106	125	155	1.18	1.44

* (Uncorr.) = total chromogen clearance

(Corr.) = true creatinine clearance

TABLE 53. Endogenous Creatinine/Inulin Clearance Ratios in Persons with Various Diseases.

Age	Sex	Diagnosis	Inulin		Creatinine		Ccr/Cin	
			Administration	Clearance	Clearance (Uncorr.)	Clearance (Corr.)	(Uncorr).	(Corr).
				ml./min	ml./min.	ml./min.		
47	F	Hypertension	S.I.*	150	101	161	0.67	1.07
35	M	Asthma	S.I.	64	76	88	1.19	1.37
40	F	Hypertension	S.I.	88	113	-	1.28	-
43	F	Hypertension	S.I.	103	94	111	0.91	1.38
64	M	Renal Calculi	S.I.	47	53	58	1.13	1.24
-	F	Hyperparathyroidism	S.I.	42	61	72	1.45	1.72
35	M	Chronic Nephritis	S.I.	69	116	151	1.68	2.18
60	M	Hypopituitarism	S.I.	49	78	100	1.59	2.02
42	F	Hypopituitarism	S.I.	78	88	109	1.14	1.40
25	M	Hypopituitarism	S.I.	39	45	84	1.16	2.16
50	M	Hypopituitarism	S.I.	57	96	128	1.69	2.24
56	M	Hypopituitarism	Infusion	99	113	149	1.14	1.50
60	M	Hypertension	Infusion	108	116	146	1.07	1.35
83	M	Hypertension	Infusion	97	115	152	1.19	1.57
76	M	Hypertension	Infusion	72	84	101	1.17	1.41
Mean							1.23	1.61

* S.I. = Single injection.

of the ratio on the actual clearance values and thus, indirectly, on renal function. Expression of the results in the form of a regression equation allows any such dependence to become apparent.

Roscoe (1953) reported that the relationship between the endogenous chromogen clearance and the inulin clearance in a series of normal subjects and those with renal disease could be expressed by the equation Ccr. = 0.76 x Cin. + 7.6. Mandel et al. (1953) reported that the relationship between the true endogenous creatinine clearance and the inulin clearance of normal subjects could be expressed by the equation Ccr. = 0.684 Cin. + 33. Josephson and Godin (1943) reported that the equation Ccr. = Cin. + 30 expressed the relationship between the exogenous creatinine clearance and the inulin clearance in a series of normal subjects and subjects with renal disease.

The interpretation of these equations, however, requires certain reservations since regression equations can be constructed to express the relationship between any two variates whether these are related or not. Unless the two variates are related the regression equation is merely a mathematical expression incapable of being interpreted. Consequently, unless there is a high degree of correlation between the variates it is unwise to place much reliance on information derived from linear regression equations.

The linear regression equations expressing the relationship between the endogenous creatinine and inulin clearances obtained in the present series of experiments, including those

of normal subjects and of subjects with renal disease, have been calculated. The linear regression of creatinine clearance on inulin clearance is Ccr. = 0.96 Cin + 39.5; that for inulin clearance on creatinine clearance is Cin. = 0.69 Ccr. - 1.5. The data and these two lines have been plotted in Fig. 14. The implications of these two equations are apparently different. The first might be interpreted as indicating the existence of a tubular component to the excretion of creatinine or inulin which is independent of the magnitude of the clearance; the second might be interpreted as indicating the existence of a tubular component to the excretion of creatinine or inulin which is proportional to the clearance. The difference between the regression lines, however, is due solely to the absence of a high degree of correlation between the two clearances.

Discussion of the Relationship between the Clearances of Creatinine and Inulin.

1. Clearance relationships of endogenous plasma creatinine level.

Smith, Goldring and Chasis (1938) found that the experimental error involved in single period clearance measurements was 8.9% and a similar figure, i.e. 7%, was reported by Ferguson et al. (1950). In the absence of change in renal function during the period of examination most of his variability can be attributed to errors in the collection of urine and to dead-space errors. Such errors are not cumulative but tend to cancel out in successive periods. The error involved in the

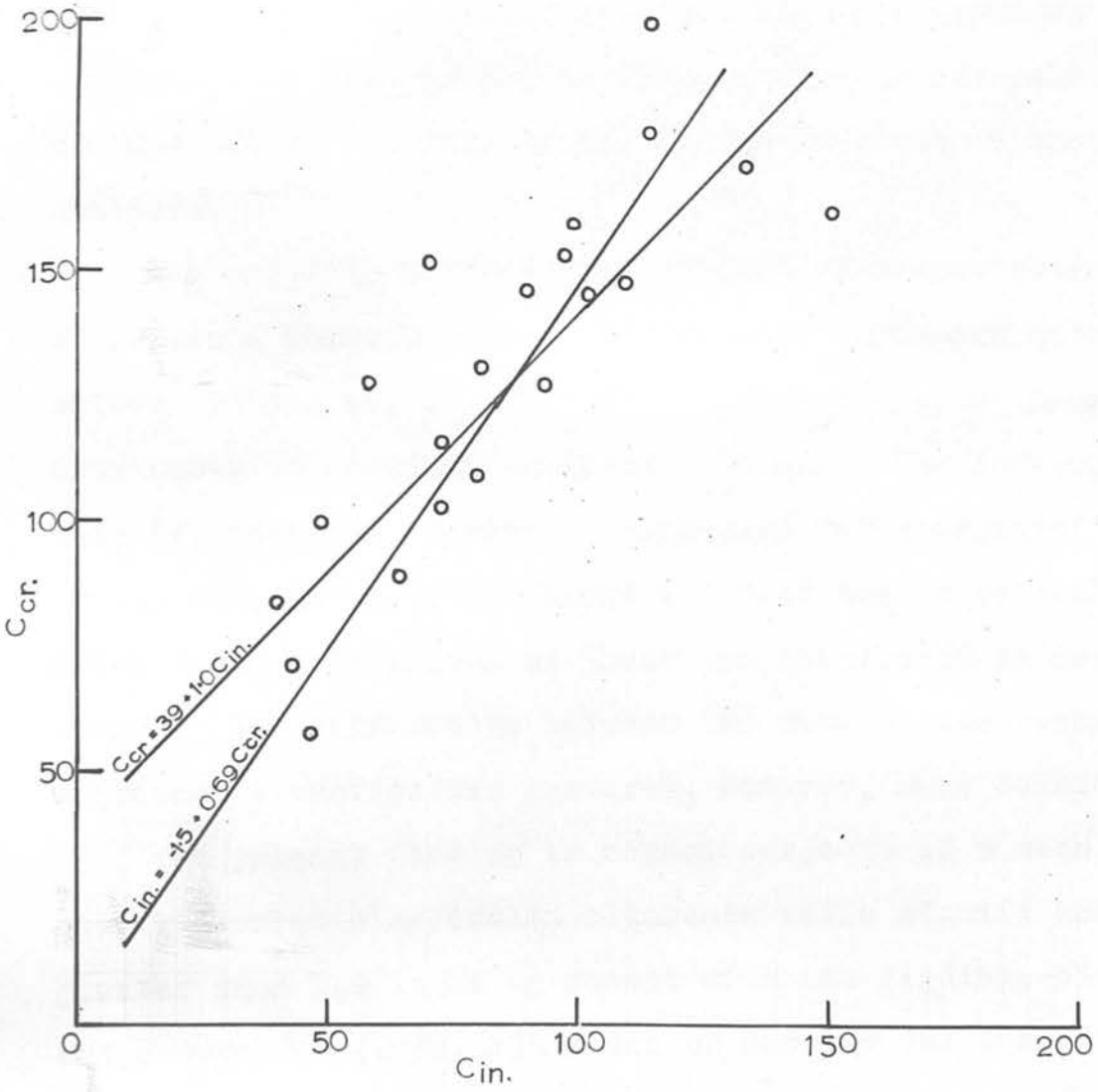

FIGURE 14. Creatinine and Inulin clearances.

Linear regression lines fitted to data from normal subjects and from those with various diseases.

estimation of clearance by averaging the results from successive periods is, therefore, considerably less than that involved in single period determinations.

Errors due to change in renal function in addition to those in the collection of urine are largely absent from the determination of the ratio of simultaneous clearances of two substances so that errors in clearance ratio determinations are due almost entirely to the errors involved in chemical analyses.

The majority of creatinine/inulin clearance ratio results of separate workers show a considerable variation within themselves (Tables 45, 46 & 47), the range being wider than can be attributed to chemical analytical errors. The inference from this is, that the excretion mechanisms for creatinine and inulin are, in fact, different and that the occasional equality noted in the clearances of these two substances is due to chance. The differences between the mean ratios observed by different investigators requires, however, some consideration.

The present finding in normal subjects of a mean endogenous true creatinine/inulin clearance ratio significantly greater than 1.0 is in agreement with the findings of Miller and co-workers (1938, 1952) and of Barclay and Kenney (1947) and, after allowance is made for the non-creatinine-chromogen, with a number of investigators who found a mean endogenous chromogen clearance rate of approximately 1.0 (Steinitz and Turkand, 1948; Brod and Sirota, 1948; Sirota et al., 1950). It is also in agreement with the conclusions of Shannon (1935) who, by extrapolation of a curve relating mean clearance ratio to creatinine plasma concentration estimated that the ratio

would be about 1.4 at endogenous levels. The present findings are contrary, however, to those of Brod and Kotatko (1949), Hare (1950), Haugen and Blegen (1953) and Mandel et al., (1953) all of whom obtained a mean ratio of approximately 1.0. Haugen and Blegen included in their series subjects with renal disease but since it is generally agreed that the clearance ratio in subjects with renal disease is greater than 1.0 the mean ratio of their normal subjects was presumably not greater than 1.0.

Different methods for the determination of creatinine in plasma and urine give different results and the absolute value of the creatinine clearance is, therefore, dependent on the procedure used. In the present investigation the method used was the same as that used by Miller and Winkler and involved the use of the NC-bacteria and "neutral" tungstic acid filtrates. On the other hand, Brod and Kotatko, Hare and Mandel et al. used Lloyd's reagent with "acid" filtrates ("acid" tungstic acid and trichloracetic acid filtrates), procedures which have been found to give plasma creatinine concentrations approximately 5% higher than those obtained with the NC-bacteria and "neutral" tungstic acid filtrates. Haugen and Blegen used Lloyd's reagent with "neutral" tungstic acid filtrates, a method which has been found to give results identical with those obtained by the method used in the present investigation. The existence in plasma of a non-diffusible fraction of creatinine would mean that the observed values for creatinine clearances are erroneously low but there is, at present, no reason to suspect that the mean relative amounts

of non-diffusible creatinine would differ in the subjects of different series. Thus, it is evident from the above considerations that while differences in the analytical procedures employed may cause slight differences in creatinine clearance measurements they can only be responsible for a small fraction of the differences between the mean clearance ratios noted by different observers.

Continuous infusions of inulin were given by Brod and Kotatko, by Haugen and Blegen and by Mandel et al. and, while it is not known for certain, presumably also by Hare. The relatively, and erroneously, high inulin clearances which are obtained shortly after single injections of inulin cannot therefore be incriminated and further, there is no evidence to suggest that the different methods which have been used for the determination of inulin in plasma and urine could be responsible for the discrepancies. However, the possibility that the absolute value of the inulin clearance is dependent on the particular preparation of inulin administered must be considered as an explanation of these findings. Indeed, Josephson and Lindahl (1943) drew attention to the fact that some preparations of inulin give anomalously low values for the inulin clearance. In considering the chemistry of inulin it was noted that a wide range of values have been reported for its molecular weight and that this variation could be due to disaggregation of fragments of a large molecule. It is conceivable that the various fragments would be excreted differently by the kidney; for example, small fragments might be readily absorbed by the tubules while extra large

molecules might be less readily filtered by the glomerular membrane so that, with inulin preparations containing significant amounts of these forms of inulin, the inulin clearance would be lower than that obtained with homogeneous inulin. There is at present, however, no means of estimating to what extent, if any, differences in the physical properties of the inulin preparation administered have been responsible for the observed discrepancies in clearance ratios.

It is, therefore, not possible to offer a single definitive explanation for the disparity between the mean endogenous creatinine/inulin clearance ratios obtained in the present series of experiments and that reported by some other workers. Assuming, however, that the present results and those of Miller and co-workers are the more correct, i.e. that the endogenous creatinine clearance is greater than the inulin clearance, a number of possible explanations can be offered. These include:- consistent errors in the chemical analyses, non-homogeneity of inulin, the presence of non-diffusible creatinine in plasma and formation of creatinine by the kidney as well as the possibility of participation of the renal tubules in the excretion of creatinine and/or inulin.

The first four of these explanations have already been considered in some detail and little further discussion is necessary. There is no conclusive evidence to confirm or exclude any of them. Inulin clearance is independent of plasma inulin concentration over a considerable range but this does not exclude tubular excretion or reabsorption of

inulin. Creatinine clearance is to some extent dependent on the plasma concentration, especially at high levels, but while this suggests tubular excretion of creatinine, it does not constitute conclusive evidence. Thus it would appear that existing evidence does not validitate the acceptance of any particular explanation for the apparent difference in the clearances of inulin and of creatinine at endogenous clearances. However further discussion of the possible relationship between these parameters is best postponed until the effect of tubular inhibitors is examined.

2. Clearance relationships at exogenous plasma levels.

The measurement of creatinine/inulin clearance ratios at exogenous plasma creatinine levels introduces sources of error additional to those involved in the determination of endogenous creatinine/inulin clearance ratios. The time-relation of the collection periods to the administration of creatinine and the plasma creatinine level obtained are important factors which must be considered in the interpretation of the results. Delay-time effects following single injections of creatinine, or following priming injections, cause clearances to be too high and arterio-venous concentration differences after oral creatinine cause clearances to be higher or lower than the true value according to whether the plasma concentration is rising or falling.

The values reported by different workers for the creatinine/inulin clearance ratios at exogenous plasma levels show a

considerable range of individual ratios. Apart from that of Hare (1950) the mean ratios are all considerably greater than 1.0, ranging from 1.25 to 1.6. Hare reported a mean ratio of 1.08 but this may have been due to the inclusion in his series of children in whom ratios of less than 1.0 have generally been found. These reports of mean exogenous creatinine/inulin clearance ratios consistently greater than 1.0 are in contrast to the frequently observed mean endogenous creatinine/inulin clearance ratios of approximately 1.0 and suggest that the mechanism for the excretion of creatinine at exogenous plasma levels is qualitatively or quantitatively different from that at endogenous levels. In view of this the scarcity of direct comparisons of creatinine clearances at exogenous and endogenous level is rather surprising.

The existence of tubular excretion of creatinine only when the plasma creatinine concentration is appreciably higher than the normal endogenous level has been suggested (Winkler and Para, 1937) as an explanation for the higher clearance ratios which have been reported at exogenous levels. However since the majority of investigators administered creatinine orally or by single injections it would seem more likely that this disparity between endogenous and exogenous clearance ratios exists as a consequence of errors resulting from the method of administration, e.g. delay-time effects. While it is possible that the exogenous clearance ratios obtained in the present series of experiments are likewise erroneously high the fact

that the endogenous creatinine clearances were seldom less than the exogenous clearances suggests that appreciable delay-time errors were not involved in the present series of experiments, in which continuous infusions of creatinine were given.

Miller and Winkler (1938) also used infusions of creatinine to obtain exogenous plasma levels but their mean value of 1.6 for the exogenous clearance ratio is somewhat suspect as a result of the short time allowed for equilibration after the priming injection. The present finding of a mean clearance ratio of 1.4 at similar plasma levels is not, therefore, inconsistent with their results.

The supposition that the exogenous clearance ratios of most workers are higher than the endogenous clearance ratios as a consequence of the method of administering the creatinine implies that the true exogenous clearance ratio is lower than the values which have been reported and, further, that the approximate agreement between the mean exogenous clearance ratio obtained in the present series of experiments (taking a figure of 1.3 as an average of the mean ratios at different levels) and those obtained by most of the other workers is purely coincidental. It is, however, quite conceivable that the factors which are responsible for disparities between the mean clearance ratios at endogenous levels also operate at exogenous levels, so that although the mean endogenous ratio obtained in the present series of experiments is higher than those reported by other workers the exogenous clearance ratios are similar to those reported by other workers as a result of the latter ratios being relatively too high for the reasons discussed above.

3. Clearance relationships in subjects with renal disease.

In subjects with functional or pathological impairment of renal function the mean endogenous creatinine/inulin clearance ratios reported are all considerably greater than 1.0 and are in approximate agreement with the present finding of a mean ratio of 1.6. It is generally believed that the higher clearance ratios found in subjects with renal disease indicate tubular excretion of creatinine and this belief receives support from data obtained by the use of tubular inhibitors (to be discussed later). If this is the true explanation for this finding, it is to be expected that in subjects with renal disease the creatinine clearances might show a more marked "self-depression" effect as the plasma concentration is increased and Miller and Winkler did, in fact, find that the exogenous creatinine/inulin clearance ratio in subjects with renal disease was less than that in normal subjects.

The higher clearance ratios in subjects with renal disease could also be due to a lowering of the inulin clearance as a result of the tubular reabsorption of inulin (or of increased tubular reabsorption of inulin if this exists normally), presumably by an active process since inulin diffuses less readily than creatinine. Examination of the relationship between the inulin clearance and the plasma concentration in subjects with renal disease, however, does not indicate any greater tendency for inulin clearance to be dependent on plasma concentration, rather the reverse (Ferguson et al., 1950; Mattar et al., 1952; Mandel et al., 1953) and, within the limits of this approach, there is no indication of active reabsorption of inulin in these subjects.

The Effect on Creatinine Clearance and on Creatinine/Inulin Clearance Ratio of Substances Influencing Tubular Activity.

In man, as in other animals, substances which are excreted or reabsorbed by the renal tubules appear in general to interfere with the tubular transport of other substances and, further, substances which are not known to be actively excreted or reabsorbed by the renal tubules also influence tubular activity. Presumably these substances act by competition for or inhibition of some enzyme system or source of energy. In order to determine whether tubular activity plays any part in the excretion of creatinine the effect of such substances on the creatinine clearance has been examined by several workers.

1. Phlorizin.

In man as in other animals the administration of phlorizin causes marked depression of all clearances due to circulatory effects and this necessitates the examination of simultaneous clearance ratios.

Phlorizin has long been known to cause glycosuria by inhibition of the tubular reabsorption of glucose. Chasis, Jolliffe and Smith (1933) and Goldring and Welsh (1934) failed to obtain any significant effect upon the creatinine/xylose clearance ratio although both clearances were depressed. However Shannon (1935), giving larger doses of phlorizin obtained a disproportionate depression of creatinine and inulin clearances so that their ratio tended to become 1.0 ($P_{cr.}$ = 10-100) and he concluded that this was evidence for the tubular excretion of creatinine. By itself

the finding could equally well constitute evidence for the re-absorption of the polysaccharide inulin.

2. Caronamide. (4-carboxy-phenyl methanesulphonate).

This substance inhibits the tubular excretion of the penicillins (Beyer, 1947), p-amino hippurate (PAH) (Borger, 1947) and p-amino salicylate (PAS) (Horne and Wilson, 1949) substances which are excreted by the renal tubules. It has no effect on the excretion of glucose, urea or amino acids (Borger, 1947).

Brod and Sirota (1948) examined its effect on endogenous chromogen clearance. In four subjects with normal renal function the mean change in chromogen clearance was +3% (-8 to +28%); the mean change in the chromogen/mannitol clearance ratio was +1 (-13 to +11%). In two subjects with renal disease the changes in chromogen clearance were -12% and -30%, respectively, and the changes in the chromogen/inulin clearance ratios were -14% and -32%, respectively.

Brod and Kotatko (1949) found that caronamide had no effect on the endogenous (true) creatinine/inulin clearance ratio in 11 subjects with normal renal function, the mean ratio before and after caronamide being 1.01. However in 12 subjects with renal disease the ratio was depressed by caronamide from a mean value of 1.58 to a mean value of 1.15.

Mattar, Barnett, McNamara and Lauson (1952) reported no change in the endogenous (true) creatinine/inulin clearance ratio in two children with the nephrotic syndrome given single or small multiple doses of caronamide, but with larger doses they found a

fall in the mean creatinine/inulin clearance ratio from 1.48 to 1.16 in three children. It is to be noted that in one subject caronamide depressed the PAH clearance to below the creatinine clearance. However, no allowance was made for protein binding of PAH which is believed to involve about 10% of the plasma PAH (Taggart, 1951).

Bucht (1949) examined the effect of caronamide on exogenous creatinine (oral) and inulin clearances. He obtained an increase in the mean inulin clearance and a decrease in the creatinine clearance so that the mean clearance ratio fell from 1.24 to 0.99. It is impossible, however, from this data to determine whether the effect was on the inulin clearance or on the creatinine clearance or on both.

3. Benemid (p-di-n-propylsulphamyl-benzoic acid).

Benemid was introduced as an inhibitor of the tubular excretion of the penicillins and subsequently it was found to be potent inhibitor of the tubular excretion of PAH and PAS (Beyer, Russo, Tilson, Miller, Verwey and Gass, 1951). It also inhibits the tubular reabsorption of uric acid (Gutman and Yu, 1951).

Sirota, Yu and Gutman (1952) reported that Benemid had no effect on the inulin clearance in subjects with gout but with normal renal function. A slight decrease was noted in the endogenous chromogen clearance, the mean clearance ratio being 1.03 before Benemid and 0.97 afterwards. Mandel et al. (1953) reported that Benemid had no effect on the endogenous (true) creatinine clearance in one normal subject and in four subjects with renal disease.

4. Diodone and PAH.

Smith et al. (1940) reported that diodone at the plasma concentration employed to measure its clearance had no effect on the endogenous chromogen clearance but at higher concentrations such as those employed in the measurement of Tm_D there was a depression of the chromogen clearance and of the chromogen/inulin clearance ratio in four normal subjects. These authors concluded that this was evidence for the tubular excretion of chromogen since the inulin clearance was unaltered.

Crawford (1948) gave large doses of diodone and PAH to subjects with normal renal function and examined the effect on the exogenous creatinine (infusion)/inulin clearance ratio. She found that while both clearances were depressed the decrease was a disproportionate one, so that the mean ratio decreased from 1.25 to 1.10.

On the other hand Brod and Sirota (1946) reported that high concentrations of PAH had no effect on the endogenous chromogen inulin ratio in subjects with renal disease and Brod and Kotatko (1949) reported that high concentrations of PAH did not effect the endogenous (true) creatinine/inulin clearance ratio in subjects with normal renal function. In subjects with renal disease, however, the ratio was slightly depressed from a mean value of 1.58 to a mean value of 1.48.

Haugen (1951) reported that high concentrations of PAH had no effect on the endogenous chromogen clearance or on the endogenous chromogen/thiosulphate clearance ratio in subjects with chronic nephritis and Mattar et al. (1952) also found no

depression of the endogenous (true) creatinine/inulin clearance ratio in five children with renal disease who were given large doses of PAH.

Thus the only significant effect of PAH on creatinine/inulin clearance ratio was that observed by Crawford at exogenous levels. If this was due to an interference of the tubular excretion of creatinine it is possible that PAH interfers with the transport of creatinine only when large quantities of the latter are being excreted by the tubules. This effect is similar to that of caronamide in normal subjects in whom caronamide only affects the creatinine/inulin clearance ratio when the creatinine concentration is artificially raised.

Summary and Conclusions.

The effect on creatinine and inulin excretion of substances influencing tubular activity may be summarised as follows.

1. Phlorizin in large doses depresses both inulin and creatinine clearances, but disproportionately so that the clearance ratio tends towards 1.0.

2. Caronamide has no effect on creatinine clearance at endogenous levels in normal subjects but depresses the creatinine/inulin clearance ratio in subjects with renal disease and in normal subjects at exogenous creatinine levels.

3. Benemid has no effect on the inulin clearance or on the endogenous creatinine clearance in normal subjects or in subjects with renal disease.

4. PAH has no effect on the clearance ratio at endogenous

plasma levels in normal subjects and a slight effect in subjects with renal disease at endogenous levels. In normal subjects at exogenous levels both PAH and diodone depresses the clearance ratio.

Large doses of phlorizin, PAH or diodone all produce depression of both inulin and creatinine clearances so that although the clearance ratios tend to become 1.0, it is impossible to determine which has been primarily affected. Caronamide and benemid do not produce disturbing circulatory effects. Any effect of these latter substances on the creatinine/inulin clearance ratio would appear to be due to changes in creatinine clearance since apart from observations of Bucht, there is no evidence suggesting that they influence inulin clearance. Bucht reported that caronamide depressed the exogenous (oral) creatinine/inulin clearance towards 1.0 by increasing the inulin clearance. Since caronamide is reported by others to have no effect on the clearance ratio in normals at endogenous levels and no effect on the inulin clearance in either normal subjects or those with renal disease, it seems possible that the findings of Bucht could have been due to an effect on the exogenous creatinine clearance which was masked by some coincidental change in renal function affecting the inulin clearance.

It may be concluded therefore that in subjects with renal disease, and possibly in normal subjects at exogenous levels, creatinine is partly excreted by tubular activity which can be partially inbihited by the administration of certain agents. This evidence does not, however, exclude the possibility that creatinine is also excreted by the renal tubules in normal subjects at endogenous levels.

IV. CONCLUSIONS REGARDING THE MEASUREMENT OF THE GLOMERULAR FILTRATION RATE IN MAN.

Conclusions Regarding the Measurement of the Glomerular Filtration Rate.

Subsequent to the general acceptance of the filtration-reabsorption theory of urine formation the problem of measurement of the rate of glomerular filtration has been one which has occupied the attention of many physiologists. It has for many years been generally believed that the glomerular filtration rate in man is of the same order of magnitude as the renal clearance of inulin or creatinine. The findings that many substances of widely different chemical composition, e.g. mannitol, allantoin and thiosulphate, have renal clearances of approximately this value and that the clearances of other substances, e.g. PAH, diodone and phenol red, tend to approach this value when the plasma concentration is sufficiently elevated, or when renal tubular activity is inhibited by various agents, have done much to substantiate this belief. In view of this some investigators have utilised the renal clearance of inulin as a measure of the glomerular filtration rate while endogenous creatinine clearance has been used by others. The latter determination has the advantage that it is a simpler procedure, requiring, in contrast to the determination of inulin clearance, neither continuous infusion nor catheterisation of the bladder.

The endogenous creatinine clearance, however, is seldom found to be identical with the inulin clearance, the former being reported to have a value somewhere between 80% and 160% of the latter and since both, when different, cannot equal the

glomerular filtration rate the possibility of a significant error in the estimation of the glomerular filtration rate becomes apparent. The glomerular filtration rate may, of course, equal neither.

For many clinical purposes even a large consistent error is unimportant. Thus, the numerical value of either clearance correlates sufficiently well with renal functional efficiency assessed on other standards, to make either test of considerable value as an index of one aspect of renal function and as such the determinations of inulin and creatinine have proved extremely useful in the investigation of abnormal renal physiology.

For the purpose of physiological research, however, in particular for the measurement of renal tubular activity, a more certain estimate of the glomerular filtration rate is desirable and in order to decide whether this can be provided, it is necessary to examine critically the present findings relative to the excretion of creatinine and inulin and those of other investigators. For this purpose the conclusions reached at each stage of this investigation are briefly summarised.

1. Inulin clearance is independent of the inulin plasma concentration over a considerable range of the latter.

2. Creatinine clearance is not entirely independent of the plasma concentration. However, if clearances at high levels are excluded from consideration the creatinine clearance is independent of the plasma concentration over a tenfold range, viz. 1 - 10 mg./100 ml.

3. The relation of the endogenous creatinine clearance to the inulin clearance is not yet clearly established but the sum of the evidence suggests that the endogenous creatinine clearance is greater than the inulin clearance in the majority of normal persons.

4. From examination of the effects of tubular inhibition it is concluded that creatinine is probably excreted by the renal tubules of persons with renal disease and possibly also in normal subjects at exogenous plasma levels.

Thus, while creatinine clearance cannot equal the glomerular filtration rate under all conditions the inulin clearance may do so. However, if the data relating to the renal clearance of creatinine at high plasma concentrations can be disregarded on the grounds that back diffusion of creatinine may occur at this plasma level, and only normal subjects considered, the preponderance of the evidence in favour of the acceptance of the inulin clearance as the nearest approximation to the glomerular filtration rate largely disappears. Both substances have clearances, which, within the experimental error are independent of the plasma concentration and any disparity between the numerical values of the clearances implies that either one or both substances are excreted and/or reabsorbed by the renal tubules, but in such a way that the tubular excretion or reabsorption is proportional to the plasma concentration, a finding which has been reported for urea and acetone (Wolf, 1951) and, over a considerable range of plasma concentrations, for xylose (Shannon, 1938).

Until a specific tubular inhibitor is discovered and shown to effect exclusively either the inulin clearance or the endogenous creatinine clearance in normal subjects the relative merit of these two clearances as measures of the glomerular filtration rate cannot be conclusively decided. However, the finding that the creatinine clearance at exogenous plasma levels of 10 mg./100 ml. is not consistently different from the endogenous creatinine clearance and the reports that the creatinine clearance at this level is depressed absolutely and relative to the inulin clearance by tubular inhibitors, perhaps suggests that creatinine is excreted by the renal tubules at endogenous levels but in such a way that the tubular inhibitors which have so far been used are unable to compete with the creatinine transport system.

The arguments so far put forward in this thesis have concerned the interpretation of experimental findings in accordance with the filtration-reabsorption theory of urine formation. It is perhaps pertinent at this stage to consider the excretion of inulin and creatinine in accordance with the diffusion theory of glomerular function for which evidence has recently been put forward by Chinard (1952).

There has been a tendency, for many years, to consider permeability of physiological membranes on an all or nothing basis so that ability of a substance to pass through the glomerular membrane has been regarded solely as a qualitative attribute. According to this concept molecules are either small enough to pass through the glomerular membrane or too large to leave

the plasma by this route and substances are, therefore, divided sharply into two groups:- those which can pass through the glomerulus and those which cannot.

According to the diffusion theory of clomerular activity the process taking place is one involving diffusion as well as mechanical filtration. Thus it is postulated that substances with small molecules, including water, pass through the glomerular membrane chiefly as a result of the hydrostatic pressure of the plasma while substances with large molecules pass through by a process of diffusion. Diffusion implies the existence of gradients of concentration and it is postulated that these occur as a result of the continuous tendency of the filtered water to reduce, by dilution, the concentration in the glomerular fluid of a substance which is diffusing from the plasma so that a synamic equilibrium is set up.

The rate at which a substance leaves the plasma, and consequently the rate at which it is excreted, depends, therefore, on the diffusibility of the substance and since the diffusibility of creatinine is approximately five times as great as that of inulin it might be expected that creatinine would escape from the plasma more readily than inulin. The disparity in the clearances of these two substances is due to their different diffusion coefficients. Further, it can be speculated that the greater disparity between the clearances in subjects with renal disease is due, at least, partly, to an alteration in the permeability of the golmerular membrane so that the latter becomes disproportionately less permeable to inulin.

Attractive though this diffusion theory may appear, there

is not, at present, sufficient evidence to warrant the rejection of the filtration theory of glomerular activity. The glomerular filtration rate must, therefore, continue to express a specific renal function, and although the mutual relationships between the renal clearances of inulin and creatinine and the glomerular filtration rate are, at present, without exact definition, one cannot deny the essential part which these parameters have played in the advancement of knowledge of renal function in health and disease.

Summary

1. The excretion of inulin in man has been examined. Over the plasma concentration range used and within the experimental error of the techniques employed the rate of excretion has been found to be directly proportional to the plasma concentration.

2. The existing methods for the determination of creatinine in plasma, serum and urine have been critically examined. It is concluded that the use of Lloyd's reagent in conjunction with precipitation of serum proteins by a method which gives 'acid' filtrates, or with diluted urines, provides the most reliable method for the determination of creatinine in these fluids.

3. The excretion of creatinine in man has been examined. With the methods employed the rate of excretion has been found to depend to some extent on the plasma concentration particularly at high plasma concentrations. Within the range of plasma creatinine concentrations from 1 to 10 mg./100 ml. the rate of excretion is independent of the plasma concentration.

4. The relationship between the clearances of inulin and creatinine has been examined. The true endogenous creatinine clearance has been found to have a variable relation to the inulin clearance, although the clearance ratio is always greater than 1.0 in normal subjects. In subjects with renal disease the clearance ratio tended to be greater than in normal subjects.

5. The possible relationship between the glomerular filtration rate and the renal clearances of inulin and creatinine have been discussed. It is concluded that there is at present no conclusive evidence to validate the acceptance of the clearance of either substance as a measure of the glomerular filtration rate. For most present day clinical purposes either may be used as an index of the glomerular filtration rate.

Appendix

Methods used in the Statistical Analysis of Results

Variates $x_1, x_2, x_3 \ldots\ldots x_n$. $y_1, y_2, y_3, \ldots\ldots y_n$.

Standard deviation (S.D.) = $$\frac{\Sigma(x^2) - \frac{(\Sigma x)^2}{n}}{n-1}$$

Standard error (S.E.) = $$\frac{S.D.}{\sqrt{n}}$$

Significance of difference between means - Students t-test,

where $$t = \frac{\overline{x}_1 - \overline{x}_2}{(S.E._1)^2 + (S.E._2)^2}$$

Values for probability (p) were obtained from tables given by Fisher (1944).

Correlation coefficient $$\frac{\Sigma xy - \Sigma x \Sigma y}{(n-1)(S.D.x)(S.D.y)}$$

Linear regression equation $$y = a + bx$$

$$b = \frac{n\Sigma xy - \Sigma x \Sigma y}{n\Sigma(x^2) - (\Sigma x)^2}$$

$$a = \frac{\Sigma y - b\Sigma x}{n}$$

Variance of y for any value of x $$V_y = \frac{\Sigma(y - y_e)^2}{n-2}.$$

where y_e is the estimated value of y for a particular value of x. For practical purposes the following derived formula was used $$V_y = \frac{n\Sigma(y)^2 - (\Sigma y)^2 - b^2\left[n\Sigma x^2 - (\Sigma x)^2\right]^2}{n(n-2)}$$

Variance of b $$V_b = \frac{n V_y}{n \Sigma x^2 - (\Sigma x)^2}$$

Variance of a $$V_a = \frac{V_y}{n-2}$$

Variance of Y where the point (x, Y) is distant from the mean point $(\bar{x}, \bar{Y})$

$$V_y = V_y \left[\frac{1}{n-2} + \frac{n(x-\bar{x})^2}{n \Sigma x^2 - (\Sigma x)^2} \right]$$

Significance of difference between estimated regression coefficients and given values. Students t-test.

b from β $$t = \frac{(b-\beta)}{\sqrt{V_b}}$$

a from α $$t = \frac{a-\alpha}{\sqrt{V_a}}$$

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